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FATTY ACID BIOSYNTHESIS AND THE INCORPORATION, CHAIN ELONGATION AND POSITIONAL DISTRIBUTION OF EXOGENOUS FATTY ACIDS IN THE MEMBRANE POLAR LIPIDS OF *ACHOLEPLASMA LAIDLAWII* STRAIN B

by

©

YUJI SAITO

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

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EDMONTON, ALBERTA

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled FATTY ACID BIOSYNTHESIS AND THE INCORPORATION, CHAIN ELONGATION, AND POSITIONAL DISTRIBUTION OF EXOGENOUS FATTY ACIDS IN THE MEMBRANE POLAR LIPIDS OF *ACHOLEPLASMA LAIDLAWII* STRAIN B submitted by YUJI SAITO in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

The existence of a fatty acid de novo biosynthetic pathway in Acholeplasma laidlawii B (formerly Mycoplasma laidlawii B) was confirmed by demonstrating the production of radioactive long-chain fatty acids (even- and odd-numbered straight-chain saturated acids) from exogenous radioactive acetate and propionate, respectively. This organism could also utilize exogenous even- and odd-numbered short-chain methyl-branched carboxylic acids for the production of the corresponding long-chain iso- and anteiso-branched fatty acids. The chain length of fatty acids synthesized by the de novo pathway was altered slightly in response to changes in growth temperature but was unaffected by the incorporation of cholesterol into the membrane. However, the uptake of exogenous fatty acids with relatively high melting points, such as 16:0, caused the preferential production of shorter-chain fatty acids; incorporation of low-melting fatty acids, such as 9-18:1c, resulted in the preferential biosynthesis of longer-chain fatty acids. In the presence of some exogenous fatty acids this organism excreted a significant fraction of its biosynthesized fatty acids into the growth medium.

This organism could systematically regulate the extent of incorporation of exogenous fatty acids into the membrane lipids in response to their physical, but not their chemical, properties. Fatty acids having moderate melting points were most extensively incorporated into the membrane lipids.

This organism could regulate the extent of chain elongation of exogenous fatty acids primarily in response to their physical nature,

although the chain elongation system also exhibited an intrinsic chain-length specificity. Generally speaking, exogenous fatty acids having lower melting points were elongated more extensively than ones having higher melting points. Alterations in both the growth temperature and the amount of cholesterol present in the growth medium did not significantly affect the extent of chain elongation. However, the simultaneous incorporation of fatty acids having high melting points, such as 16:0, reduced the extent of elongation of the other exogenous fatty acids; incorporation of exogenous fatty acids with low melting points, such as 9-18:1c, increased the extent of elongation of the other fatty acids.

The enzyme systems responsible for fatty acid biosynthesis and for the incorporation and chain elongation of exogenous fatty acids all appear to function so as to maintain the fluidity of the lipids of the plasma membrane of this organism within a certain broad range.

The positional distribution of a variety of fatty acids in two neutral glycolipids was studied. Fatty acyl groups capable of the closest molecular packing have the strongest affinity for the 1-position. The positional specificities of a series of positional isomers of cis-octadecenoic acid in phosphatidylglycerol were also investigated. The affinity of the isomers for the 1-position was increased as the position of the double bond was moved from the carboxyl to the methyl end of the fatty acid molecule, with the exception of several fatty acids having the double bond close to a carboxyl group. It was also demonstrated that the positional distribution of any particular fatty acid was not fixed entirely by its own chemical or physical nature, but rather was flexible, depending upon the total fatty acid composition of the membrane polar lipids.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to my supervisor, Dr. Ronald N. McElhaney, for his constant guidance and encouragement throughout the course of this study.

I am also indebted to Dr. P. G. Barton not only for many valuable discussions, but also for his generosity in providing me with the series of positional isomers of *cis*-octadecenoic acid.

I would also like to thank Ms. Bonnie Gordashko for her technical assistance and Mr. John Silvius for his technical assistance as well as for his many helpful suggestions and discussions.

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Finally, I wish to extend my gratitude to my parents, Atsumi and Shizue Saito, for their encouragement and understanding during my graduate studies.

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LIST OF ABBREVIATIONS

ACP or ACP-SH	acyl carrier protein
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BCCP	biotin carboxyl carrier protein
BSA	bovine serum albumin
C	curie
CDP-DG	cytidine diphosphate diglyceride
CoA or CoA-SH	Coenzyme A
cpm	counts per minute
CTP	cytidine triphosphate
DGDG	diglucosyl diglyceride
DGMG	diglucosyl monoglyceride
FAD	flavine adenine dinucleotide
FFA	free fatty acid
GLC	gas-liquid chromatography
GPDGDG	glycerophosphoryldiglucosyl diglyceride
LPG	lyso-phosphatidylglycerol
MGDG	monoglucosyl diglyceride
MGMG	monoglucosyl monoglyceride
m, μ, n	milli, micro, nano (10^{-3}, 10^{-6}, 10^{-9})
MW	molecular weight
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide reduced form
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate reduced form

LIST OF ABBREVIATIONS (continued)

O-PG	O-amino acyl ester of phosphatidylglycerol
PA	phosphatidic acid
PG	phosphatidylglycerol
RAM	radioactivity monitoring system
TLC	thin-layer chromatography
t-RNA	transfer ribonucleic acid
UDP	uridine diphosphate
Short-hand notation of fatty acids	The number before the colon represents the total number of carbon atoms in the fatty acid molecule and the number after the colon represents the number of double bonds; the position of the double bond(s) or the cyclopropane ring in the fatty acid is given by the number before the hyphen, which denotes the first carbon atom bearing the functional group in question as numbered from the carboxyl end of the molecule. The *cis* and *trans* geometrical configurations of the double bond(s) and cyclopropane ring are abbreviated as c and t, respectively; the presence of iso-methyl- and anteiso-methyl-branching, and a cyclopropane ring in a fatty acid molecule are indicated by the letters i, ai and cp, respectively.

Materials and General Methods

1. Organism: The organism used throughout these studies was *Acholeplasma laidlawii* (formerly *Mycoplasma laidlawii*), strain B, originally obtained from D.G. ff. Edward (Wellcome Research Laboratories, Beckenham, Kent, England).

2. Radioactive fatty acids and their sodium salts: Sodium acetate (2:0)-1,2-C^{14} (54 mC/mmole), sodium propionate (3:0)-1-C^{14} (22 mC/mmole) and sodium butyrate (4:0)-1-C^{14} (11.5 mC/mmole) were purchased from New England Nuclear (Boston, Mass., U.S.A.). Sodium hexanoate (6:0)-1-C^{14} (10mC/mmole) and octanoic acid (8:0)-1-C^{14} (3.87 mC/mmole) were obtained from ICN, Isotope & Nuclear Division (Irvine, Calif., U.S.A.). Decanoic acid (10:0)-1-C^{14} (21 mC/mmole) was purchased from Amersham/Searle (Des Plaines, Ill., U.S.A.). Dodecanoic acid (12:0)-1-C^{14} (57.5 mC/mmole), Tetradecanoic acid (14:0)-1-C^{14} (48 mC/mmole), and hexadecanoic acid (16:0)-1-C^{14} (9.6 mC/mmole) were products of Applied Science Laboratories Inc. (State College, Penna., U.S.A.). The radiopurity was greater than 99 per cent for all of these materials.

3. Nonradioactive fatty acids: The nonradioactive fatty acids were obtained from one of the following companies: Analabs (North Haven, Conn., U.S.A.), Nu-Chek-Prep Inc. (Elysian, Minn., U.S.A.), Serdary Research Laboratories (London, Ontario, Canada). The purity was greater than 99 per cent for all the acids used. Positional isomers of octadecanoic acid were synthesized and generously donated by Dr. P.G. Barton, Department of Biochemistry, The University of Alberta.

4. Enzymes: The lipase from Rhizopus arrhizus delemer was the generous gift of Mr. T. Tomita (Tanabe Seiyaku Co. Ltd., Tokyo, Japan).

5. Silicic acid: Bio-Sil A, 200-325 mesh (Biorad Co., Richmond, Calif., U.S.A.) was used for the initial column chromatographic purification of the total lipids. Unisil, 200-325 mesh (Clarkson Chemical Company Inc., Williamsport, Penna., U.S.A.) was used for the column chromatographic separation of the total membrane lipids into various fractions. Silica gel H (E. Merk, Germany) was used for thin-layer chromatography (TLC).

6. Other chemicals used were all reagent grade.

7. Preparation of lipid-poor growth medium: 120 g of tryptose (Difco Laboratories, Detroit, Mich., U.S.A.) was dissolved in 1200 ml of hot water, cooled and acidified to pH 1 to 2 with about 50 ml of conc. HCl. The solution was twice extracted with 300 ml of chloroform to remove most of the lipoidal material present. 22.2 g of Tris (buffer) (J. T. Baker Chemical Company, Phillipsburg, N.J., U.S.A.) was added, water was then added to bring the total volume to 6 l, and the pH was adjusted to 8.0 to 8.2 with 40 per cent NaOH. To 1 l of the sterilized tryptose medium were added 10 ml of 25 per cent ($^w/v$) glucose, 1 ml of a 100,000 units/ml solution of Penicillin G (Pfizer, Montreal, Quebec) and 40 ml of 10 per cent ($^w/v$) BSA (bovine serum albumin), B grade, fatty acid-poor (Calbiochem, San Diego, Calif., U.S.A.), which reduces the lytic effect of exogenous fatty acids. Glucose was added as a sterile aqueous solution after autoclaving. The sterile Penicillin G was prepared by injecting sterilized water

into a vial containing crystalline Penicillin G. The pH of the BSA solution was adjusted to 8.0 to 8.2 by addition of NaOH, and the solution was sterilized by Seitz filtration.

8. Conditions of growth: Fatty acids were added to the lipid-poor growth medium as sterile ethanolic solutions, containing 10 mg or more of fatty acids per ml of ethanol. The final concentration of ethanol in the growth medium was 0.5 per cent or less. Cells were grown statically at 34 to 37°C unless otherwise specified. Cell growth was monitored by measuring the absorbancy at 450 nm.

9. Extraction of lipids from the cell membrane: Lipids were extracted by the Bligh and Dyer method (1959) throughout these studies. The cell pellet obtained by the centrifugation (13,000 x g, 15 min) of 1 l of culture medium was tritulated with 40 ml of water, mixed with 150 ml of methanol-chloroform (2:1), and centrifuged (650 x g, 15 min). The supernatant was removed and retained, and the cell pellet was extracted a second time. The combined one-phase chloroform-methanol-water solution was mixed well with 200 ml of chloroform, 200 ml of water, and 400 ml of chloroform in that order, and centrifuged (650 x g, 15 min). This centrifugation resulted in a clear separation into two phases. The upper methanol-water phase and the interphase contained non-lipid materials like proteins, and the lower chloroform phase contained essentially all the lipids present in the cells. The upper phase and the interphase were eliminated by aspiration.
After reducing the volume by evaporation, the lower chloroform layer was passed through a chromatographic column prepared from 5 g of

silicic acid in chloroform. The column was washed once with 100 ml of methanol. The volume of solvents used at each step of the extraction procedure was altered proportionally when the volume of culture medium used was less than 1 l.

10. Preparation of methylesters of fatty acids: Methylesters of fatty acids were prepared from complex polar lipids by transesterification by heating with acidified anhydrous methanol according to the method of Gander _et_ _al_. (1962). The lipids were dried in a screw-capped test tube (15 x 1.5 cm) with teflon liner. To the test tube were added 10 ml of methanol and several drops of conc. sulfuric acid, and the tube was heated at 65°C for 2 hours. After cooling, the contents of the tube were transferred to a 125-ml separatory funnel, mixed with 20 ml of water, and the methylesters were extracted twice with 10 ml of hexane. The extracted hexane solution was dried with anhydrous sodium sulfate. A smaller scale extraction method was also successfully used, especially when the volume of the culture medium was less than 250 ml. A smaller screw-capped test tube (10 x 1.2 cm), again with a teflon liner, was used. The esterification was done in 1 ml of methanol and one drop of conc. sulfuric acid at 65°C for 2 hours. After cooling, 2 ml of water and 0.5 ml of hexane were added, and the small test tube was shaken well. The upper hexane layer was transferred to a conical tube with a Pasteur pipette, and the lower phase was extracted once more with 0.5 ml of hexane. To the conical tube containing the hexane extracts were added a very small amount of anhydrous sodium sulfate, and the tube was quickly centrifuged in a

clinical centrifuge. The supernatant hexane extracts were injected into the gas-liquid chromatograph (GLC) either directly or after concentration by evaporation.

11. Analysis of fatty acid composition by gas-liquid chromatography (GLC): The gas-liquid chromatograph used in these studies was a Hewlett Packard model 5700A (San Diego, Calif., U.S.A.) equipped with hydrogen flame ionization detectors, a model 7128A strip chart recorder, and a model 3370B electronic integrator. The chromatographic column (stainless steel, ¼" x 6') was packed with 10 per cent diethylene glycol succinate on Anakrom ABS, 70/80 mesh, support (Analabs, North Haven, Conn., U.S.A.). Chromatographic conditions were as follows: column temperature, initial - 80 to 120°C, final - 210°C; temperature raise, 4 or 8°C/min; carrier gas, helium; flow rate, 60 to 100 ml/min; range of sensitivity, 100 or 1000. Fatty acids were identified on the basis of retention times as compared to known standards. The proportion of each fatty acid present in a sample was calculated from the area under each peak as determined by the electronic integrator.

12. Analysis of the radioactivity of fatty acids by the radioactivity monitoring gas-liquid chromatography (RAM-GLC) system: The GLC was equipped with a splitter capable of shunting variable proportions of the gas leaving the chromatographic column into the flame ionization detector and the RAM system (Nuclear Chicago Co., Des Plaines, Ill., U.S.A.). The RAM system consisted of three components — a combustion furnace control (model A-6034), a counter module (model A-6035) and a single channel ratemeter (model 8731).

The strip chart recorder used for the GLC analyses was also connected to the RAM system. The sensitivity ranges used were 100,000 or 300,000 cpm, and the time constant was 1 second. Propane was used as a quench gas and its flow rate was 30 to 40 per cent that of the helium carrier gas. The radioactivity of the fatty acids was calculated from the areas under each peak as determined by triangulation. Thus the RAM-GLC system provided a simultaneous analysis of the radioactivity and the mass of each fatty acid.

CHAPTER I

DE NOVO FATTY ACID BIOSYNTHESIS IN ACHOLEPLASMA LAIDLAWII B

Introduction

In this introduction, I wish to present a general review of the biosynthesis of the two most common groups of fatty acids found in living organisms, namely the even-numbered, straight-chain saturated and unsaturated fatty acids. Since unsaturated fatty acids often are derived from their saturated analogues, the biosynthesis of the saturated fatty acids will be discussed first. Prior to 1958 it was thought that fatty acid biosynthesis in animal cells was accomplished by a reversal of the fatty acid beta-oxidation pathway known to be present in mitochondria. It is now clear that the *de novo* biosynthetic pathway is a completely distinct enzyme system and differs from the *beta*-oxidation system in the following ways:

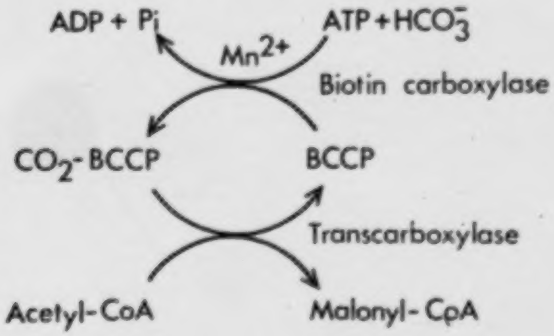
1. ACP (Acyl Carrier Protein) functions as the intermediate carrier throughout the biosynthetic sequence, whereas CoA (Coenzyme A) is the intermediate carrier in *beta*-oxidation.

2. Malonyl ACP provides the two carbon units for the *de novo* synthesis, whereas acetyl CoA is liberated in the *beta*-oxidation scheme.

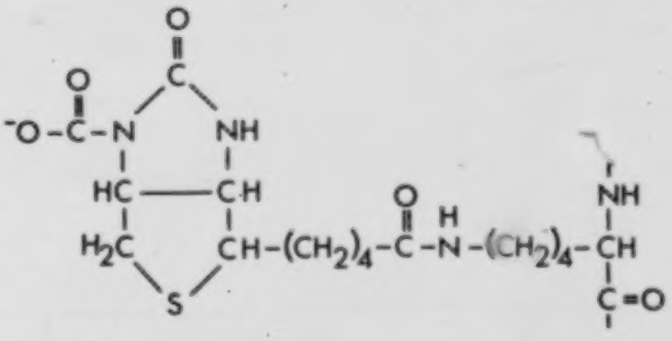
3. During biosynthesis D-*beta*-hydroxy acids are produced, whereas during *beta*-oxidation, L-*beta*-hydroxy acids are formed.

4. During biosynthesis NADPH, and possibly NADH, are utilized as the hydrogen donors, whereas during *beta*-oxidation NAD and FAD are used as the hydrogen acceptors.
5. The *beta*-oxidation enzymes are localized in the mitochondria, whereas the enzymes of *de novo* biosynthesis are found in the supernatant fraction of animal cells.

The *de novo* synthesis of saturated fatty acids is catalyzed by two enzyme systems which function sequentially, the acetyl-CoA carboxylase and the fatty acid synthetase complex. Acetyl-CoA carboxylase catalyzes the carboxylation of acetyl-CoA to malonyl CoA, which in turn acts as the two-carbon donor for the chain elongation of a primer molecule, typically acetyl-ACP. The acetyl-CoA carboxylase of *Escherichia coli* has been resolved into three functionally distinct protein components, BCCP (biotin carboxyl carrier protein), biotin carboxylase, and transcarboxylase. The reactions catalyzed by these enzymes are seen in the following scheme (Alberts and Vagelos, 1968; Alberts *et al.*, 1969; Fall and Vagelos, 1972):

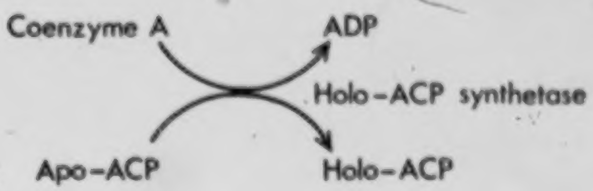


The prosthetic group of BCCP, biotin, is carboxylated in a Mn^{2+}- and ATP-dependent reaction catalyzed by biotin carboxylase to form CO_2^--BCCP. The third protein component, the transcarboxylase, catalyzes the transfer of the carboxyl group from CO_2^--BCCP to acetyl-CoA to form malonyl-CoA; this reaction regenerates BCCP which is then available to accept another carboxyl group. The prosthetic group, biotin, is known to be covalently bound to the epsilon-amino group of a lysine residue in the protein moiety of BCCP molecules, and can accept carbonate to form 1'-N-carboxyl biotinyl BCCP:

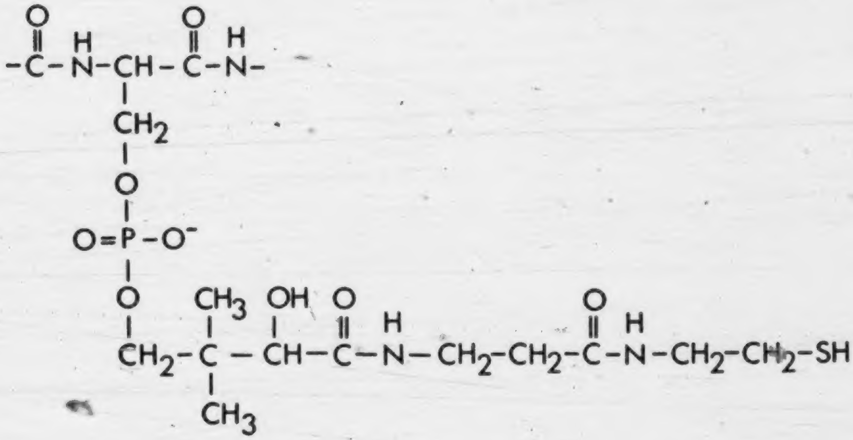


Animal and yeast acetyl-CoA carboxylases have not been successfully separated into functional components.

The second enzyme system, which catalyzes the synthesis of saturated long-chain fatty acids from malonyl-CoA and a suitable primer, is called the fatty acid synthetase complex. This system exists as a multi-enzyme complex which has never been separated into its active components in yeast and animal cells; however, the individual protein components of the fatty acid synthetases of E. coli, Clostridium kluyverii, and some plants are found not to be associated when these cells are disrupted. The individual enzymes of the fatty acid synthetase complex have thus been isolated and the details of the intermediate reactions elucidated by using these latter organisms. However, whether the component proteins exist *in vivo* as a multienzyme complex in these bacterial and plant systems is not known. *Euglena gracilis*, grown in the light on mineral media, and *Mycobacterium phlei* are rather exceptional in that they appear to contain both types of fatty acid synthetase (Delo *et al.*, 1971; Brindley *et al.*, 1969). A central component of the fatty acid synthetase complex is a small, acidic protein called Acyl Carrier Protein (ACP) (Majerus and Vagelos, 1967). It contains 4'-phosphopantetheine as the prosthetic group and is synthesized from CoA by the following transfer reaction (Majerus *et al.*, 1965; Elovson and Vagelos, 1968):

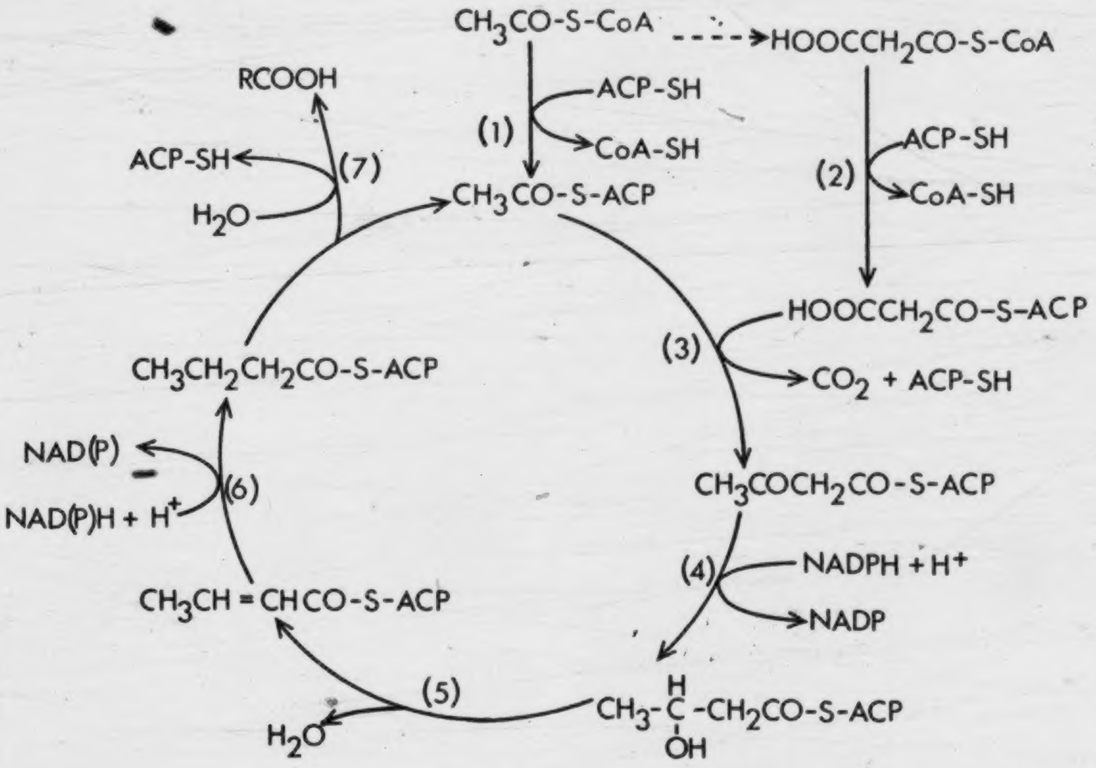


The complete amino acid sequence of *E. coli* ACP has been determined, and the prosthetic group is known to be linked to a serine residue of the protein through a phosphodiester linkage (Vanaman *et al.*, 1968):



The thiol group of 4'-phosphopantetheine can form a high energy thiol ester with fatty acids, and this thiol ester serves as the active intermediate in the various biochemical reactions catalyzed by the synthetase complex. ACP, or a protein functioning like ACP, has been identified not only in E. coli, but also in various other biological systems capable of the de novo synthesis of fatty acids.

The entire sequence of the synthesis, and the names of the enzymes responsible for each step, are shown in the following map:


CH3CO-S-CoA
HOOCCH2CO-S-CoA
ACP-SH
(1)
CoA-SH
CH3CO-S-ACP
(2)
ACP-SH
CoA-SH
HOOCCH2CO-S-ACP
(3)
CO2 + ACP-SH
CH3COCH2CO-S-ACP
(4)
NADPH + H+
NADP
H
CH3-C-CH2CO-S-ACP
OH
(5)
H2O
CH3CH = CHCO-S-ACP
(6)
NAD(P)H + H+
NAD(P)
CH3CH2CH2CO-S-ACP
(7)
H2O
ACP-SH
RCOOH

1. Acetyl-CoA-ACP transacylase
2. Malonyl-CoA-ACP transacylase
3. beta-ketoacyl-ACP synthetase (condensing enzyme)
4. beta-ketoacyl-ACP reductase
5. beta-hydroxy-ACP dehydrase
6. Enoyl-ACP reductase
7. Fatty acyl-ACP hydrolase

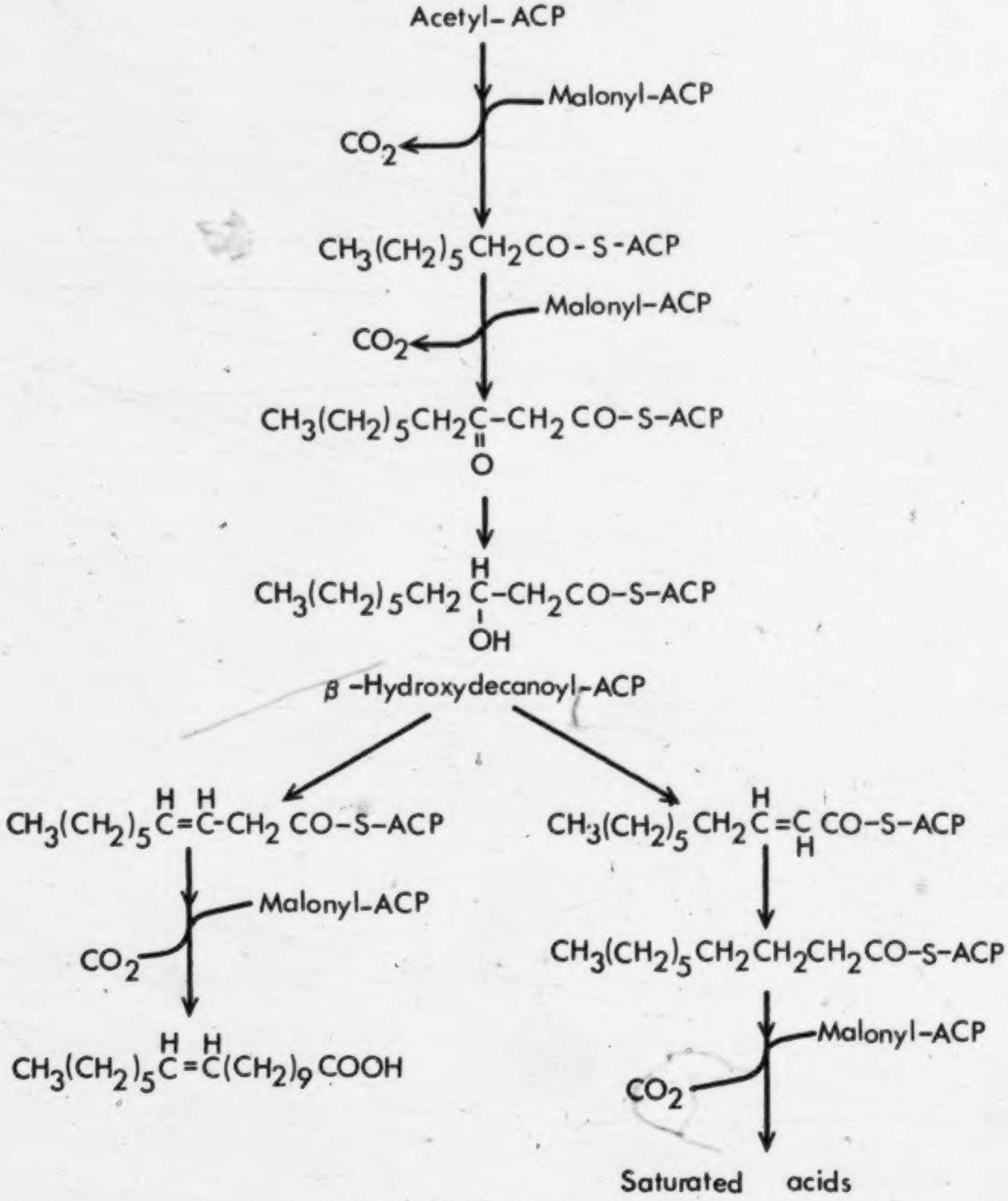
Both acetyl-CoA and malonyl-CoA are converted to corresponding ACP thiol esters by reactions 1 and 2, respectively. Acetyl-ACP and malonyl-ACP condense (reaction 3), and liberate ACP and carbon dioxide from acetyl-ACP and malonyl-ACP, respectively. The beta-keto acyl-ACP is then reduced by NADPH (reaction 4) to form beta-hydroxy acyl-ACP. This hydroxy compound is then dehydrated (reaction 5) to crotonyl ACP, which is further reduced by NADPH or NADH (reaction 6) to butyryl-ACP, a compound two carbon units longer than the primer, acetyl-ACP. This cycle repeats until the acyl chain reaches the appropriate length, at which time the fatty acyl-ACP is hydrolyzed to a free fatty acid (reaction 7). The detailed mechanisms for the component reactions of yeast and animal fatty acid synthetases, which exist as non-dissociable multi-enzyme complexes, have not been elucidated. Although some minor differences between these two systems are known to exist (Kumar *et al*., 1972), the overall reaction scheme is expected to be very similar.

Both the acetyl-CoA carboxylase and fatty acid synthetase complexes are known to be subject to biochemical controls, through both allosteric regulation and adaptive change in enzyme content. Although allosteric regulation has been demonstrated *in vitro* with tricarboxylic acids, long-chain fatty acyl CoA's and phosphorylated sugars, it is still unclear whether these regulatory mechanisms also operate *in vivo*. Adaptive changes in enzyme content in response to various nutritional states, however, have been clearly demonstrated *in vivo* by immunochemical techniques (Majerus and Kilburn, 1969). The levels of acetyl-CoA carboxylase and fatty acid synthetase are markedly higher in the livers of animals maintained on fat-free diets as compared to starved animals, due to both the increased rate of synthesis and the decreased rate of degradation of these enzyme systems.

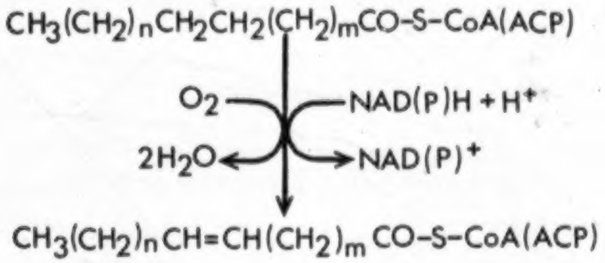
Straight-chain saturated fatty acids containing an even number of carbon atoms are often the only major products of the de novo fatty acid synthetic pathway. Some organisms, however, can synthesize straight-chain saturated fatty acids containing an odd number of carbon atoms by utilizing propionyl-CoA as a primer instead of acetyl-CoA, presumably by the same sequence of reactions previously discussed (Horning et al., 1961; Kaneda, 1963). Other organisms can also synthesize even- or odd-numbered isobranched fatty acids and/or odd-numbered anteisobranched fatty acids by using isobutyryl-, isovaleryl-, and alpha-methyl butyryl-CoA, respectively, as primers (Horning et al., 1961; Kaneda, 1963). These CoA derivatives are derived from the amino acids, valine, leucine, and isoleucine, respectively, by transamination followed by oxidative decarboxylation.

There exist two different pathways for the biosynthesis of unsaturated fatty acids, the anaerobic and aerobic pathways. The former is found only in members of the class Eubacteriales. There are no organisms known to have both the anaerobic and aerobic pathways. Since the anaerobic pathway is most closely related to the de novo fatty acid synthetic pathway, this pathway is discussed first. Hofmann et al. (1959) found that cis-unsaturated fatty acids having 12 to 16 carbon atoms, all having the double bond at the same distance from the methyl terminus of the molecule as cis-vaccenic acid, can serve as growth factors replacing cis-vaccenic acid in Lactobacilli, and suggested the existence of an anaerobic pathway for unsaturated fatty acid synthesis, which involves the chain elongation of preformed shorter-chain unsaturated fatty acids.

Goldfine and Bloch (1961) reported that cultures of *Clostridium butyricum* extended exogenous radioactive C8 and C10 saturated acids to labeled longer-chain monounsaturated as well as saturated acids, whereas no unsaturated acids arose from substrates longer than C10. The enzyme or enzymes responsible for the synthesis of unsaturated fatty acids in this organism must specifically attack only certain short-chain fatty acid intermediates. The crude fatty acid synthetase from *E. coli* was shown to contain an enzyme that catalyzed the dehydration of *beta*-hydroxydecanoyl-ACP, a normal intermediate in the fatty acid synthesis, to a mixture of *trans*-2- and *cis*-3-decenoate; this enzyme also catalyzes the interconversion of the two isomers (Norris *et al.*, 1964). *Trans*-2-decenoyl-ACP is a normal intermediate of fatty acid synthesis, and can be reduced by enoyl-ACP reductase to the saturated decanoyl-ACP and further elongated. *Cis*-3-decenoate, however, is different from the normal intermediate in the configuration and the position of the double bond, and is elongated without being reduced. This *cis*-double bond is, therefore, retained and long-chain unsaturated fatty acids are produced by further chain elongation. The enzyme, *beta*-hydroxydecanoyl thioester dehydrase, has now been purified to homogeneity, and its mode of action has been studied (Kass *et al.*, 1967; Brock *et al.*, 1967). Since *E. coli* unsaturated fatty acid auxotrophs, which lack *beta*-hydroxydecanoyl thioester dehydrase (Silbert and Vagelos, 1967), can still synthesize the long-chain saturated fatty acids, this enzyme must not be required for the synthesis of long-chain saturated fatty acids. In fact, altogether three other *beta*-hydroxy-ACP dehydrases have now been found to be involved in saturated fatty acid biosynthesis in *E. coli*. They are *beta*-hydroxybutyryl-, *beta*-hydroxyoctanoyl-, and *beta*-hydroxypalmityl-ACP dehydrase, and have a specificity toward short-, intermediate-, and long-chain beta-hydroxy-acyl-ACP's, respectively (Mizugaki et al., 1968a, 1968b). All of these dehydrases, however, produce only the normal trans-2-enoyl intermediates and are essential for the biosynthesis of long-chain saturated fatty acids. The following scheme shows the biosynthetic pathways for both saturated and unsaturated fatty acids.


Acetyl- ACP
Malonyl-ACP
CO_2
$CH_3(CH_2)_5CH_2CO-S-ACP$
Malonyl-ACP
CO_2
$CH_3(CH_2)_5CH_2C(=O)-CH_2CO-S-ACP$
$CH_3(CH_2)_5CH_2CH(OH)-CH_2CO-S-ACP$
β -Hydroxydecanoyl-ACP
$CH_3(CH_2)_5CH=CH-CH_2CO-S-ACP$
Malonyl-ACP
CO_2
$CH_3(CH_2)_5CH=CH(CH_2)_9COOH$
$CH_3(CH_2)_5CH_2CH=CHCO-S-ACP$
$CH_3(CH_2)_5CH_2CH_2CH_2CO-S-ACP$
Malonyl-ACP
CO_2
Saturated acids

All organisms, with the exception of certain bacteria, form unsaturated fatty acids by the aerobic pathway, which involves the direct introduction of a double bond into the hydrocarbon chain of preformed fatty acids by an oxygen-mediated abstraction of hydrogen, as shown in the following overall reaction (Bloch, 1969):


$CH_3(CH_2)_nCH_2CH_2(CH_2)_mCO\text{-}S\text{-}CoA(ACP)$
O_2
$NAD(P)H + H^+$
$2H_2O$
$NAD(P)^+$
$CH_3(CH_2)_nCH{=}CH(CH_2)_mCO\text{-}S\text{-}CoA(ACP)$

Oxygen and NADPH (or NADH) act as electron acceptor and electron donor, respectively. The reaction is apparently catalyzed by a series of enzymes, which transfer electrons from reduced pyridine nucleotide to oxygen by way of flavoproteins and nonheme-iron proteins (Nagai and Bloch, 1968). These enzymes exhibit positional- and stereospecificity toward the hydrogens to be eliminated and only produce the _cis_-isomers (Bloch, 1969). For the oxidative insertion of double bonds, fatty acyl thioesters are required substrates. CoA derivatives are active in the desaturation systems of yeast, animal tissues, and _M. phlei_; plant systems use the ACP thioesters. Gurr _et al_. (1969) and Talamo _et al_. (1973), however, have suggested that even the fatty acids esterified to lecithins can be a substrate for the desaturation reaction. The monounsaturated acids formed can be either elongated (in thioester form) or be further desaturated to higher polyunsaturated acids by an enzyme system (or systems) which also requires oxygen and reduced pyridine nucleotide. The question of whether the same enzyme system is involved in these subsequent desaturations as in the synthesis of monounsaturated acids remains to be answered.

It is known that the chain length of the fatty acids synthesized depends on the location of the fatty acid synthetase complex. The apparent chain length of fatty acids in lipids may be different depending upon the tissue or organ examined, or even depending upon the membrane system in the same cell (Van Deenen, 1966). Many investigators have been interested in the mechanism for the termination of fatty acid chain elongation. Greenspan _et_ _al_. (1970) undertook a detailed study of substrate specificity of the _beta_-ketoacyl-ACP synthetase, which is responsible for the first condensing reaction in the elongation cycle, and demonstrated the importance of this enzyme in the termination of chain elongation and in the accumulation of specific saturated and unsaturated fatty acids in the cell. Fatty acyl-ACP hydrolase, which is the enzyme responsible for the liberation of the free fatty acid products from their ACP derivatives, may also function in determining the chain length by specifically hydrolyzing ACP derivatives having the appropriate chain lengths. Simoni _et_ _al_. (1967) demonstrated that some property of the ACP itself might control the chain length of the fatty acid products. Although ACP components from plants and _E_. _coli_ functioned interchangeably in their respective fatty acid synthetase systems, the chain length of the fatty acids produced varied according to the source of the ACP added. In spite of these investigations the precise mechanism of the chain termination is still not fully understood, and remains to be elucidated in future studies.

Rottem and Razin (1967) reported the incorporation of sodium acetate-1,2-C^{14} into polar lipids by washed cells of Acholeplasma laidlawii (oral strain). Pollack and Tourtellotte (1967) also reported the formation of long-chain radioactive fatty acids by A. laidlawii B from radiolabeled acetate, and separated these fatty acids into their molecular species by reversed-phase Thin Layer Chromatography. Radioautography of the TLC chromatoplates revealed that 14:0, 16:0, 18:0 and possibly 15:0 were radiolabeled although quantitative results were not obtained due to the insensitivity of the methods used. No radiolabeled unsaturated fatty acids were detected.

Rottem and Panos (1970) developed a cell-free system from A. laidlawii A for the synthesis of long chain fatty acids. As is often noted in cell-free systems, the pattern of fatty acids formed was shifted toward the longer-chain fatty acids, with 18:0 being the predominant fatty acid produced (83.2 per cent), whereas 14:0 and 16:0 are the predominant products observed in vivo. In this study it was clearly shown that there was no synthesis of unsaturated fatty acids; only saturated fatty acids were formed when beta-hydroxydecanoic acid replaced acetate in this fatty acid synthetase system. The addition of a beta-hydroxy thioester dehydrase preparation from wild type E. coli B to this cell-free system resulted in the formation of both saturated and unsaturated acids. Recently a partial purification of ACP from Mycoplasmas and Acholeplasma was reported (Rottem et al., 1973).

In this chapter the author will attempt to determine conclusively whether or not the de novo biosynthetic pathway is present in the simple procaryotic microorganism Acholeplasma laidlawii strain B (formerly Mycoplasma laidlawii B) and to characterize the products of this pathway. He will also investigate the primer specificity of the fatty acid biosynthetic system in this organism. In addition, alterations in the nature and quantity of the fatty acids synthesized by this pathway, in response to changes in growth temperature and to the presence of various exogenous fatty acids and cholesterol in the growth medium, will be studied. Finally, some conclusions as to the biochemical mechanisms underlying de novo fatty acid biosynthesis and its regulation will be advanced, and the possible biological significance of these mechanisms will be discussed.

A. The *de novo* biosynthesis of fatty acids by *Acholeplasma laidlawii* B

1. Materials and Methods

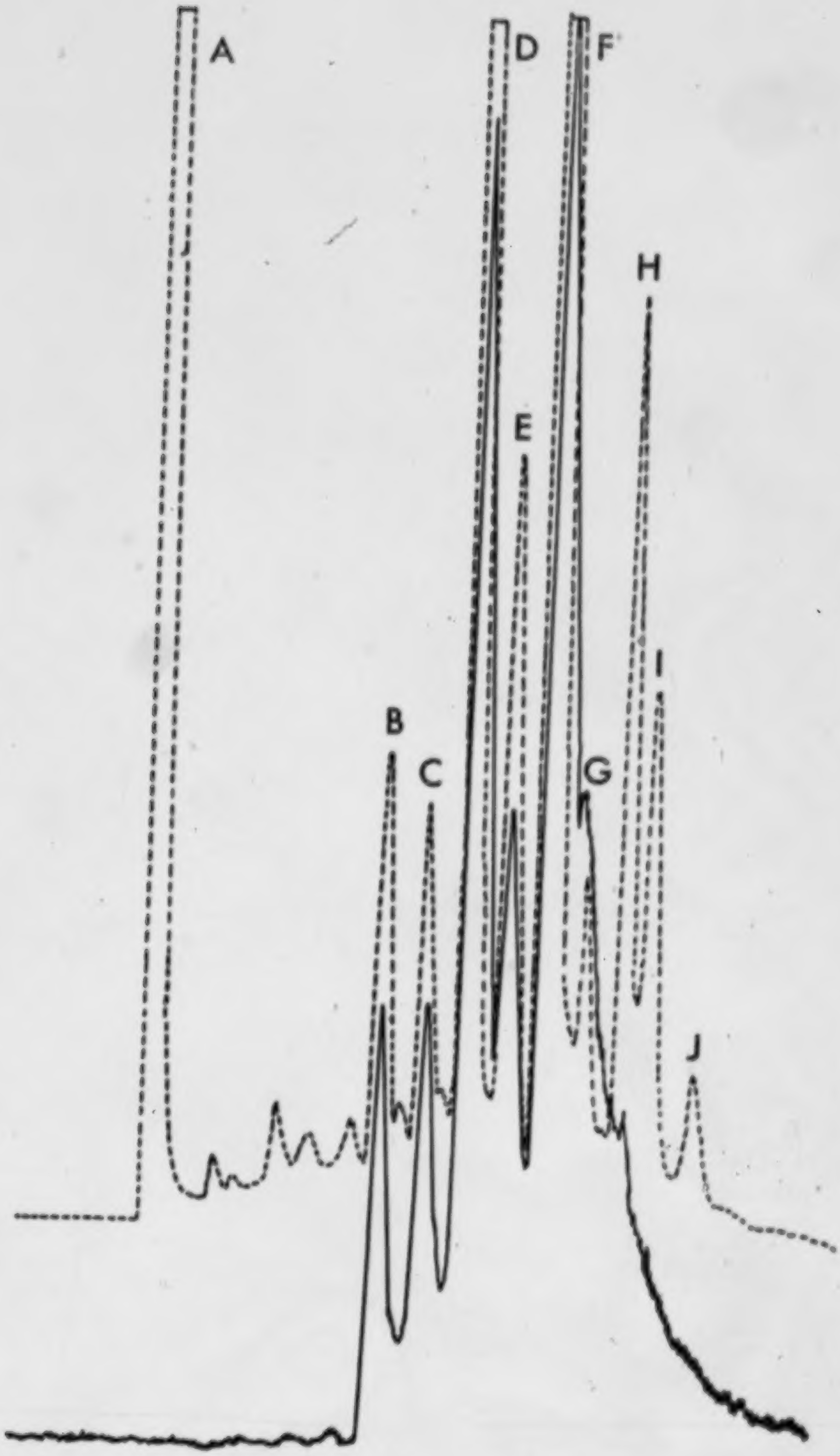
Sodium acetate-1,2-C^{14} (500 μC, 9.25 μmole) or sodium propionate-1-C^{14} (50 μC, 2.27 μmole) were added to 125 ml of lipid-poor growth medium before inoculation. Cells were harvested in late-log phase after 20 hours of growth. Lipids were extracted and methyl esters were prepared by the small scale procedure.

2. Results

Fatty acids containing less than twelve carbon atoms have been reported to be absent from the membrane lipids of *A. laidlawii* B (McElhaney and Tourtellotte, 1969). However, the significance of this observation has been questioned, since conventional procedures for the transesterification and recovery of fatty acids may result in extensive loss of the relatively volatile short-chain fatty acids and their esters. To test definitively whether short-chain fatty acids are lost from *A. laidlawii* B membrane lipids during the conventional preparation of methyl esters, hexanoic acid (6:0)-1-C^{14} was utilized as a short-chain fatty acid standard. Hexanoate radioactivity was measured with a liquid scintillation counter (Beckman, Model LS-230) at each step of the transesterification procedure: before and after 2 hours of incubation in acidic methanol at 65°C, after cooling and extraction of the reaction mixture with hexane, and after removal of the hexane solvent with a rotory evaporator. Hexanoic acid and its methyl ester were quantitatively retained in the acidic methanol solution during the 2 hour incubation at 65°C, and methyl hexanoate was also quantitatively extracted into the hexane phase. However, almost 90 per cent of the fatty acid methyl ester was lost during evaporation of the hexane solvent. This loss was partially prevented by using diethylether instead of hexane, and by evaporation of solvent under a stream of nitrogen gas instead of by use of a rotary evaporator. However, the loss was still more than 50 per cent with this modified procedure. The simplest way to overcome this problem of evaporative loss was to use the smallest possible volumes of methanol and hexane in the esterification and ester extraction steps, respectively. If the RAM-GLC system is operated at high sensitivity settings, it is even possible to analyze the fatty acid composition directly from the hexane extracts without any evaporation of the hexane solvent. Methyl esters of fatty acids from A. laidlawii B membrane lipids from a 250 ml culture were prepared using various volumes of acidic methanol. After the incubation water was added to bring the total volume to 10 ml, the esters were extracted by the conventional method and analyzed by gas-liquid chromatography (GLC). With 1 ml of methanol, the smallest volume tested, quantitative recovery of esters of fatty acids with more than twelve carbon atoms was obtained. The volume of hexane required to quantitatively extract esters from 1 ml methanol plus 2 ml water was then determined, and quantitative recovery of methyl esters was obtained using two extractions with 0.5 ml hexane each. Using this small-scale transesterification procedure, no fatty acid containing less than 12 carbon atoms were detected in the total membrane lipids of A. laidlawii B, indicating that indeed short-chain fatty acids are absent from the lipids of this organism.

Figure 1 shows the RAM-GLC chromatogram of fatty acids obtained from cells grown in the presence of sodium acetate-1,2-C^{14}.

Fig. 1 .   A RAM-GLC chromatogram illustrating the incorporation of 1,2-C^{14}-acetate into the fatty acids of the total membrane lipids of *A. laidlawii* B. The cells were grown in the presence of 1,2-C^{14}-acetate (500μC, 9.25μmoles) in 125 ml of the lipid-poor growth medium and harvested in late-log phase. Membrane lipids were extracted and methylesters were prepared by the procedures described in the text. The fatty acid composition and the radioactivity associated with each fatty acid component were determined as described in the text. Identification of the major components: A, solvent; B, 12:0; C, 13:0; D, 14:0; E, 15:0; F, 16:0; G, 17:0; H, 18:0; I, 9-18:1c; J, 9,12-18:2c,c. ------- and ——— are GLC (mass) and RAM (radioactivity) tracings, respectively. This is a representative chromatogram from triplicate experiments.


A
B
C
D
E
F
G
H
I
J

Radioactive peaks corresponding to 12:0, 13:0, 14:0, 15:0 and 16:0 are clearly demonstrated, and less obvious peaks for 17:0 and 18:0 are also observed on the shoulder of the 16:0 peak. No radioactivity is associated with the unsaturated fatty acids, 9-18:1c and 9,12-18:2cc. These two unsaturated fatty acids must then represent exogenous fatty acids derived from the growth medium. In fact, these unsaturated fatty acids were found to be the major residual fatty acids in the lipid-poor growth medium, along with 16:0 and 18:0.

From the RAM-GLC chromatograms, the relative specific activities for each fatty acid synthesized from acetate were calculated by the following equation:

$$\text{Relative specific activity} = \frac{\text{Radioactivity}}{\text{Mole Fraction}}$$

The relative radioactivity of each fatty acid was taken as the area under the appropriate RAM peak. The mole fraction of that acid was obtained by dividing the area under the GLC peak by the molecular weight of the fatty acid methyl ester. The relative specific activity, therefore, gives the relative radioactivity per molecule in arbitrary units. Although the absolute specific activity (µC/µmole) can be obtained by carefully calibrating the RAM-GLC system, determination of the absolute specific activity on a routine basis would have been time-consuming, and would have yielded little additional useful information.

If the relative specific activities of a series of biosynthetically-related fatty acids (even- or odd-numbered saturated fatty acids, for example) are plotted against the chain length of the fatty acid produced, a single straight line should be obtained provided that these acids are synthesized from a single primer by the sequential addition of two-carbon units derived from acetate. Moreover, the intersect on x-axis should indicate the chain length of the (unlabeled) primer for that series of fatty acids. Figure 2 clearly indicates that even-numbered and odd-numbered saturated fatty acids are synthesized from radioactive acetate and "cold" propionate, respectively, under these experimental conditions.

Odd-numbered straight chain fatty acids are, generally speaking, rather rare in living organisms. It is therefore of interest to note that this organism produces about 10 to 20 mole per cent of these fatty acids under these growth conditions. Since odd-chain saturated fatty acids are not detected in the residual fatty acids of the lipid-poor growth medium, they must indeed be synthesized de novo with propionate serving as the primer, as has been demonstrated in other systems (Wakil, 1970). This was confirmed by growing cells in the presence of sodium propionate-1-C^{14}. The results of this experiment are presented in Figure 3. There are no radioactive peaks for the even-numbered fatty acids, although considerable activity is incorporated into odd-numbered fatty acids. The relative specific activities of the odd-numbered fatty acids were calculated from the data presented in this figure. The ratio of the relative specific activities of 13:0 to 15:0 is 1.06, which is reasonably close to the ratio of 1.00 expected if both fatty acids were formed exclusively from propionate. However, the question of whether the propionate is produced by the cell or incorporated from the medium remains to be answered.

Table 1 shows the fatty acid compositions of cells grown in sodium acetate, sodium propionate, or without fatty acid supplementation. The compositions are quite similar in each case, except for

Fig. 2 . The relationship between the relative specific activity and the chain length of the saturated fatty acids biosynthesized by *A. laidlawii* B. Cells were grown in the presence of 1,2-C^{14}-acetate, and both the fatty acid composition and the radioactivity associated with each fatty acid were simultaneously determined by RAM-GLC, as illustrated in Fig. 1 . The relative specific activity of each fatty acid was calculated as described in the text. ——— and -------- are the series of fatty acids containing an even and odd number of carbon atoms, respectively. The values presented are the averages of triplicate experiments. The variation of the relative specific activity values for each fatty acid is indicated by vertical bars. However, the absissa intercept values were very similar in each of the three experiments.

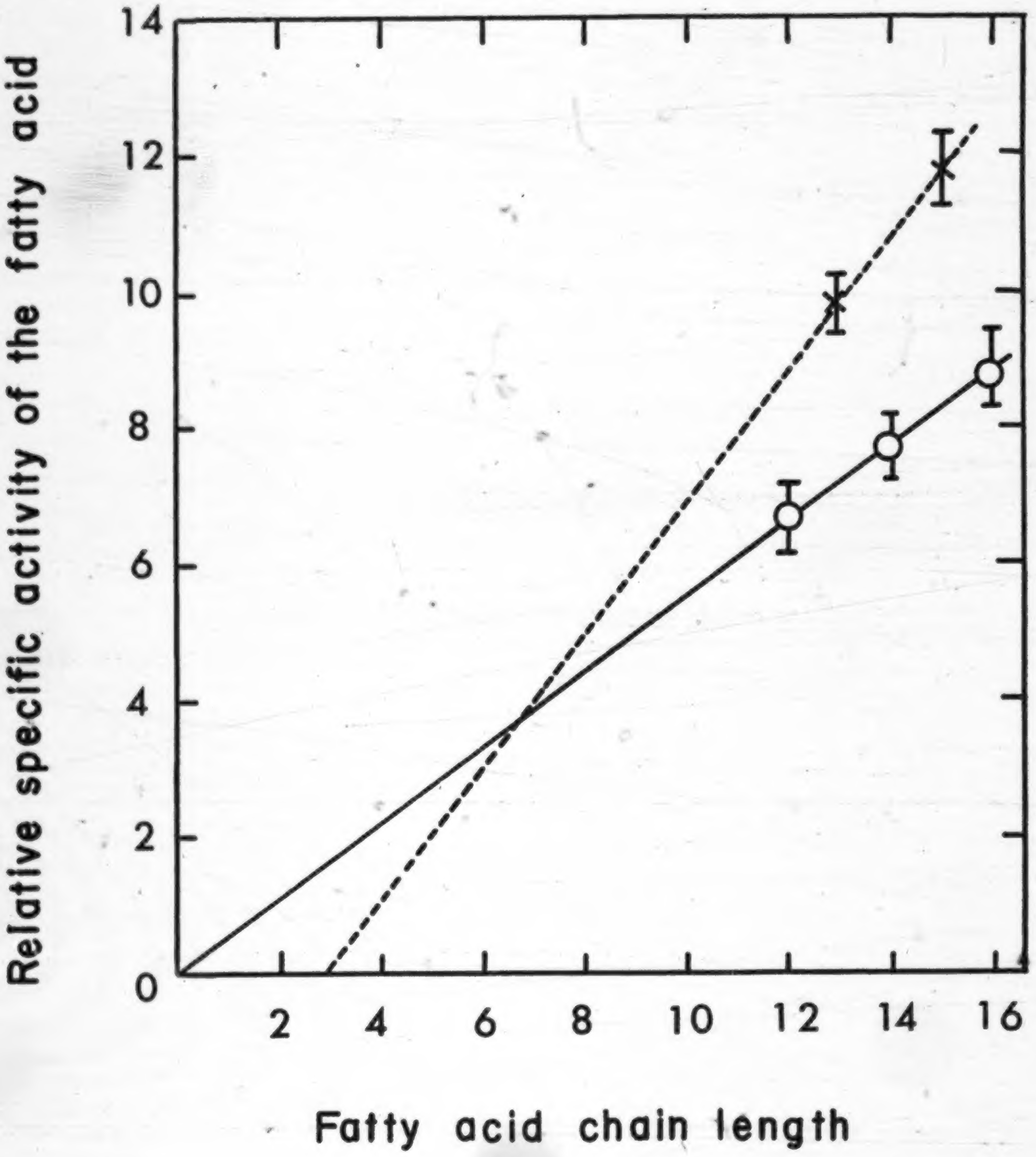

Relative specific activity of the fatty acid
0
2
4
6
8
10
12
14
2
4
6
8
10
12
14
16
Fatty acid chain length

Fig. 3. A RAM-GLC chromatogram illustrating the incorporation of 1-C^{14}-propionate into the fatty acids of the total membrane lipids of *A. laidlawii* B. The cells were grown in the presence of 1-C^{14}-propionate (50μC, 2.27μmoles) in 125 ml of the lipid-poor growth medium and harvested in late-log phase. Membrane lipids were extracted and methylesters were prepared by the procedures described in the text. The fatty acid composition and the radioactivity associated with each fatty acid were simultaneously determined by RAM-GLC, as described in the text. Identifications: A, solvent; B, 12:0; C, 13:0; D, 14:0; E, 15:0; F, 16:0; G, 17:0; H, 18:0; I, 9-18:1c; J, 9,12-18:2c,c. ---------- and ——— are the GLC (mass) and RAM (radioactivity) tracings, respectively. This is a representative chromatogram from triplicate experiments.

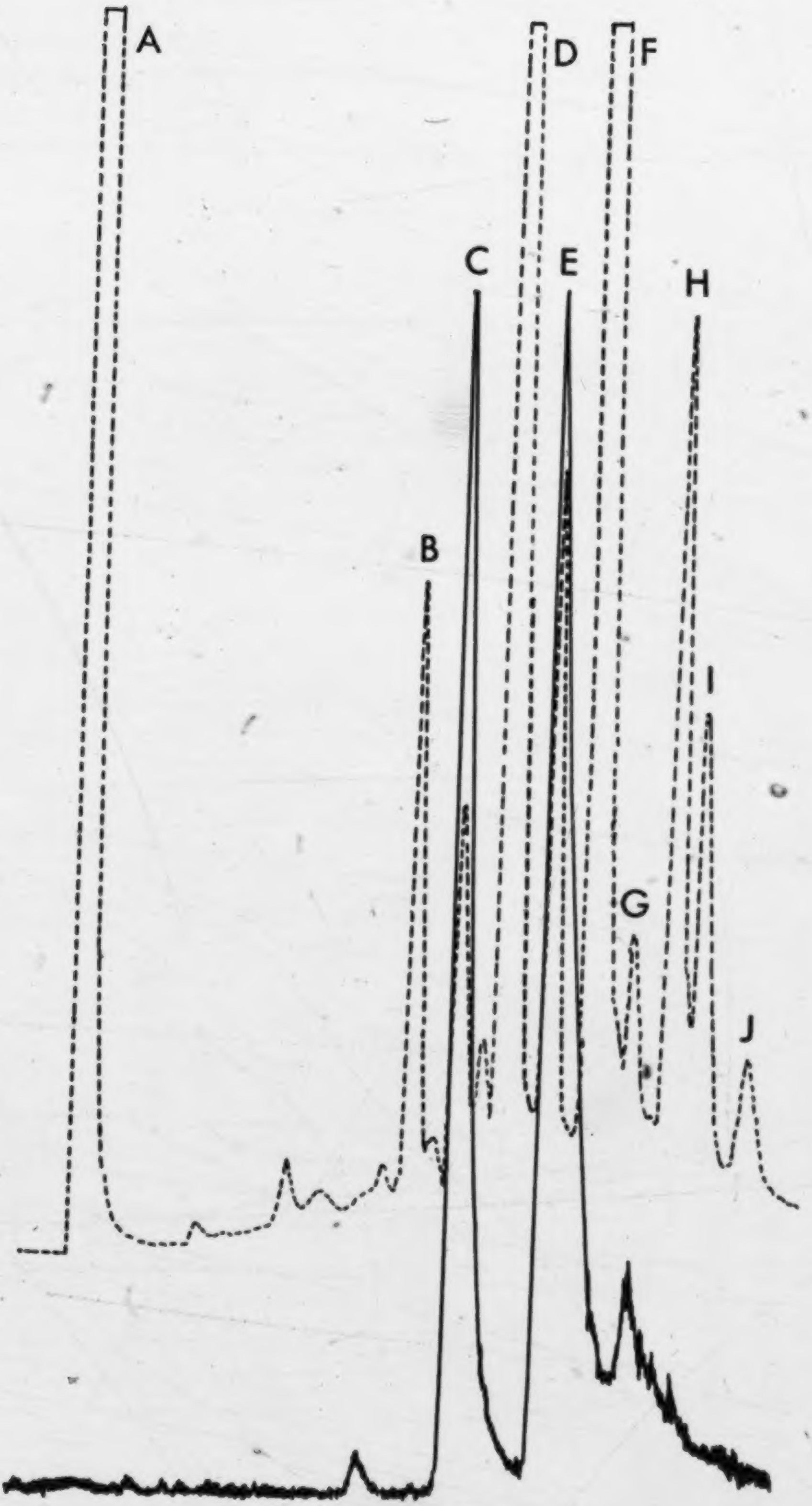

A
B
C
D
E
F
G
H
I
J

Table 1 . The fatty acid composition of the total membrane lipids of A. laidlawii B grown with exogenous sodium acetate or sodium propionate

Fatty acid found	Exogenous acids		
	none	sodium acetate	sodium propionate
12:0	7.4[a]	5.4	6.6
13:0	3.1	5.0	3.8
14:0	34.2	24.2	31.2
15:0	5.1	9.0	6.7
16:0	36.5	37.3	36.6
17:0	1.6	3.3	1.3
18:0	6.5	9.1	7.0
18:1	4.5	5.0	5.1
9,12-18:2c,c	1.3	1.7	1.8

[a] The fatty acid compositional values are expressed in mole per cent and are representative values from triplicate experiments. The fatty acid composition of the total membrane lipids of A. laidlawii B grown with sodium acetate and sodium propionate were calculated from the GLC chromatogram seen in Figs. 1 and 3, respectively.

the lower concentration of 14:0 noted for cells grown in the presence of sodium acetate. The exceptionally low level of 14:0 is probably due to over-growth in this particular experiment (Knivett and Cullen, 1967; Panos and Rottem, 1970). The fatty acid composition observed for sodium propionate-grown cells is very similar to that obtained with cells grown in the lipid-poor growth medium without fatty acid supplementation. Although propionate is incorporated and elongated as is shown in Figure 3, propionate is apparently not incorporated in large enough quantities under these conditions to change the overall fatty acid composition by elevating odd-numbered fatty acid levels. The major fatty acids synthesized de novo are 14:0 and 16:0, with each comprising about 30 to 40 mole per cent of the total esterified fatty acids. Lesser amounts, 5 to 10 mole per cent, of 12:0 are also synthesized. Odd-numbered fatty acids, particularly 15:0, are also produced by the de novo biosynthetic pathway, and comprise 10 to 20 mole per cent of the total fatty acids.

These experiments clearly show that acetate and propionate can be used by A. laidlawii B as primers for the biosynthesis of even- and odd-numbered saturated fatty acids, respectively. It is, then, of interest to examine whether this organism could utilize any other types of exogenous shorter-chain fatty acids as primers for de novo biosynthesis. Short-chain iso- and anteiso-branched carboxylic acids can also serve as primers for the synthesis of their longer-chain homologues, as is illustrated in detail in Table 2. However, if a short-chain carboxylic acid contains a cis-double bond, or more than one methyl branch, it can not serve as a primer. Thus acrylic acid (propenoic acid), beta-methylcrotonic acid, 3,3-dimethylbutyric

Table 2 The fatty acid composition of the total membrane lipids of A. laidlawii B grown in the presence of various methyl-branched, short-chain fatty acids at 1.2 mM concentrations

Fatty acid found	Exogenous fatty acid 4:0i	5:0i	5:0ai
12:0i	0.2[a]	-	-
12:0	0.9	0.3	0.7
13:0i	-	17.2	-
13:0ai	-	-	3.4
13:0	0.3	-	-
14:0i	43.2	-	-
14:0	4.5	2.9	3.7
15:0i	-	41.5	-
15:0ai	-	-	45.9
15:0	3.5	-	-
16:0i	17.1	-	-
16:0	19.1	9.8	14.1
17:0i	-	13.2	-
17:0ai	-	-	15.1
17:0	1.4	-	-
18:0i	0.8	-	-
18:0	3.5	3.8	4.4
18:1	4.3	8.7	9.2
18:2	1.3	2.7	3.2

---

[a] All fatty acid compositional values are expressed in mole per cent and are the average of triplicate experiments.

acid, and 2,2-diethylacetic acid are not utilized as primer molecules by the de novo fatty acid biosynthetic system of this organism.

3. Discussion

The plots of relative specific activity versus the chain length of the odd- and even-numbered saturated fatty acids synthesized from exogenous radioactive acetate indicate that both classes of fatty acids are synthesized de novo from (radioactive) acetate and (nonradioactive) propionate, respectively (Fig. 2). Since we would expect comparable utilization of radiolabeled acetate (in the form of the malonyl-ACP thioester) for the chain elongation of both even- and odd-numbered fatty acids, the plot described above would be expected to yield two parallel straight lines. However, an inspection of Figure 2 reveals that these lines are not parallel, but that the increase in the specific activity with chain length is significantly more marked for the odd-chain fatty acids. There are two possible explanations for this observation. The first possibility is that the "lipid-poor" growth medium may still contain appreciable amounts of even-numbered, exogenous fatty acids, and that *A. laidlawii* B incorporates significant quantities of these nonradioactive acids into the membrane lipids. If the pattern of incorporation of these exogenous fatty acids is roughly similar to the pattern of de novo biosynthesis, then the apparent specific activity of the even-numbered fatty acids would be reduced in comparison to the odd-chain saturated fatty acids. Indeed, an analysis of the lipid-poor growth medium does reveal the presence of even-numbered fatty acids and an absence of odd-chain fatty acids (Table 3). Another observation favoring this possibility is the

Table 3. The fatty acid composition of the "lipid-poor" growth medium

Fatty acids found	mole %	mg/100ml
10:0	7.4[a]	0.07
12:0	4.5	0.05
14:0	10.3	0.13
16:0	15.6	0.22
18:0	20.4	0.32
9-18:1c	30.8	0.48
5,9-18:2c,c	11.0	0.17

[a] All values presented in this table are representative of duplicate experiments.

very low specific activity of stearic acid noted in other RAM-GLC chromatograms (not presented) where this peak is well-resolved from the shorter-chain saturated fatty acids, which indicates that 18:0 is derived primarily from the growth medium.

The second explanation for the increased specific activity of odd-chain fatty acids may be due to changes in differential rates of biosynthesis of odd- and even-numbered fatty acids with time. Since this organism produces large amounts of acetate from the glycolysis of glucose, the specific activity of exogenous radioactive acetic acid is continually being reduced by dilution with nonradioactive, endogenous acetate as cell growth proceeds. If we assume that the propionic acid, which functions as the primer for odd-chain fatty acid _de novo_ biosynthesis, is derived from a limited amount of this compound in the growth medium, then the synthesis of odd-chain saturated fatty acids may occur only at the early stages of growth, when the specific activity of acetate, and thus of the endogenous fatty acid products, is relatively high. Due to the progressive depletion of exogenous propionate, only even-numbered saturates of lower specific activity may be produced at later stages of growth, thus resulting in a generally reduced average specific activity for this class of fatty acids. Whatever the explanation for the lower specific activity of the even-numbered saturated fatty acids, it is clear that most of the 12:0, 14:0 and 16:0 found in the membrane lipids of this organism is produced by _de novo_ biosynthesis from an acetic acid primer.

It is evident from Table 2 that _A. laidlawii_ B can successfully use not only acetate and propionate primers for _de novo_ biosynthesis, but also isobutyrate, isovalerate, and _alpha_-methylbutyrate for the synthesis of even- and odd-number isobranched and odd-numbered anteisobranched fatty acids, respectively. This organism apparently is able to convert these short-chain carboxylic acids to their corresponding CoA derivatives through the action of their acyl-CoA synthetase, and are also presumably converted to their corresponding acyl-ACP derivatives by one or more acyl-CoA:acyl-ACP transacylases. Butterworth and Bloch (1970) studied the primer specificity for *in vitro* fatty acid synthesis by *Bacillus subtilis* and *E. coli* fatty acid synthetase fractions. They found that *B. subtilis* lacked acetyl-CoA: acetyl-ACP transacylase, so that it could only utilize acetyl-ACP, but not acetyl-CoA, for the synthesis of straight-chain saturated fatty acids. In fact, if acetyl-ACP is provided in the enzyme reaction mixture, this compound can be efficiently utilized for the production of straight-chain saturated fatty acids, although this organism synthesizes primarily branched-chain fatty acids under normal culture conditions. They also concluded that the *E. coli* enzyme fraction as a whole utilizes branched-chain intermediates poorly, but that the transacylase specificity is not the only critical factor in this system. In the presence of a relatively high concentration (19 µM) of isobutyryl-ACP, only 70 per cent of the long-chain fatty acids produced by the *E. coli* system were isobranched acids. At the same concentration the presence of isobutyryl-CoA results in the production of only 2 per cent isobranched fatty acids. This observation suggests that the low activity or high K_m of the isobutyryl-CoA:isobutyryl-ACP transacylating enzyme is one of the critical factors in the poor synthesis of isobranched fatty acids by *E. coli* under normal culture conditions. It would be of great interest to study the utilization of various primers as free fatty acids or (as CoA- and ACP-derivatives) by a cell-free fatty acid synthetase system from *A. laidlawii* B.

B. The effect of exogenous fatty acids on *de novo* fatty acid biosynthesis

1. Materials and Methods

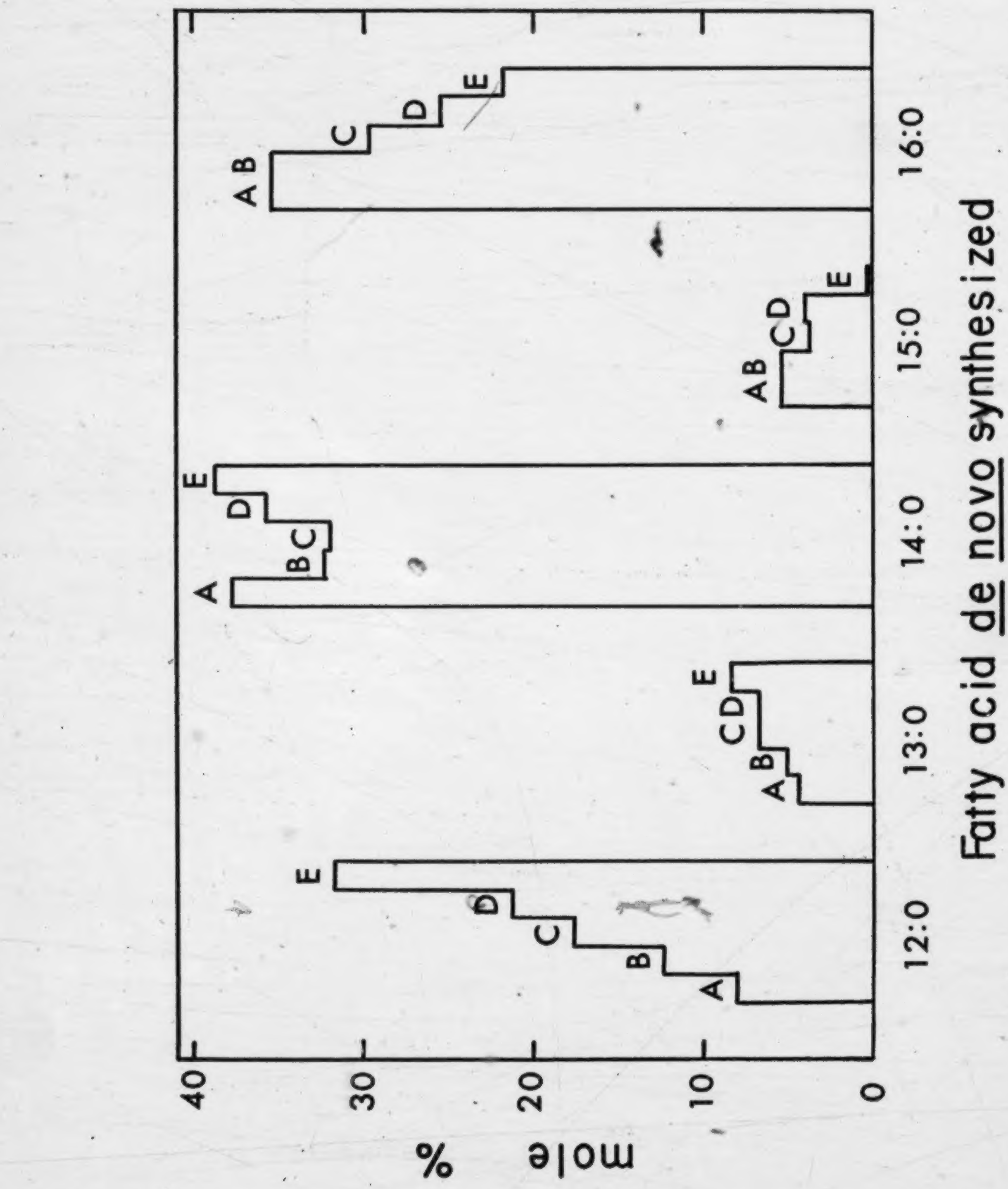
For experiments performed in the absence of radioactive acetic acid various concentrations of either 17:0 or 9-18:1c were added to 125 ml of the lipid-poor growth medium before inoculation. Cells were harvested in late-log phase and lipids were extracted and purified as described previously. Methyl esters of fatty acids were prepared by transesterification of the extracted lipids, and the fatty acid composition was analyzed by GLC. For radioactive experiments, 0.12 mM of 16:0, 17:0, or 9-18:1c were added before inoculation to 10 ml of the lipid-poor growth medium containing sodium acetate-1,2-C^{14} (62.5 μC; 0.8 μmole). Cells were harvested in late-log phase by high speed centrifugation (48,000 x g, 20 min.) to ensure that the supernatant did not contain any cell debris. The cell pellet obtained from 10 ml of the growth culture by centrifugation was then suspended in 4 ml water, and 0.1 mg of 11:0 was added to the suspension as an internal standard. Lipids were extracted from the suspension by a small scale Bligh and Dyer (1959) procedure. Lipids were also extracted from the 8 ml supernatant as described below, which is essentially a modified final step of the Bligh and Dyer procedure. Eight ml of supernatant was mixed well with 30 ml of methanol-chloroform (2:1) and to this solution were added 30 ml of chloroform, 10 ml of water, and 0.1 mg of

11:0 as an internal standard. The resulting suspension was mixed well and centrifuged (650 x g, 20 min.). The lower chloroform layer was removed and passed through a silicic acid column (1 g), and the column was then washed with 25 ml of methanol. To the eluent was added 200 µl of 5 per cent KOH in 95 per cent ethanol to convert short-chain fatty acids, if there were any, into their less volatile potassium salts, and the solution was dried. The dried lipids were transferred to small screw-capped test tubes by dissolution in small volumes of chloroform-methanol (2:1) and subsequently dried under a stream of nitrogen. Fatty acid methyl esters were prepared from the lipids extracted both from cells and growth medium, by the small scale procedure, and analyzed by RAM-GLC.

2. Results

Two different fatty acids were utilized in the experiments where radioactive acetate was not added. These fatty acids are both incorporated without being elongated (or shortened) appreciably and are not synthesized de novo significantly. The incorporation of one of them, 17:0, increases the gel to liquid-crystalline transition temperature of the membrane lipids and makes the hydrocarbon core less fluid. Incorporation of the other, 9-18:1c, acts in exactly the opposite fashion. The fatty acid compositions of the membrane lipids from cells grown in the presence of various concentrations of one of these two exogenous fatty acids were then analyzed and a quantitative analysis of the fatty acids produced by de novo biosynthesis was determined. Fig. 4 shows the effect of the incorporation into the membrane lipids of increasing

Fig. 4 . The effect of the amount of exogenous 17:0 incorporated into membrane lipids on the composition of the fatty acids biosynthesized de novo by A. laidlawii B and incorporated into the membrane lipids. Cells were grown in the presence of varying amounts of 17:0 in 125 ml of the lipid-poor growth medium. The membrane lipids were extracted from late-log phase cells and methylesters of the membrane lipid fatty acids were prepared by the procedures described in the text. The total fatty acid composition was first analyzed by GLC, as described in the text; the relative amounts (expressed in mole per cent) of the various endogenous fatty acids were then calculated by ignoring the presence of any exogenous fatty acid. The amount of 17:0 incorporated into the membrane lipids in each case was: A, 0.0; B, 19.7; C, 40.9; D, 53.3; and E, 67.5 mole per cent. All values presented here are representative of duplicate experiments.


mole %
40
30
20
10
0
A
B
C
D
E
12:0
A
B
C D
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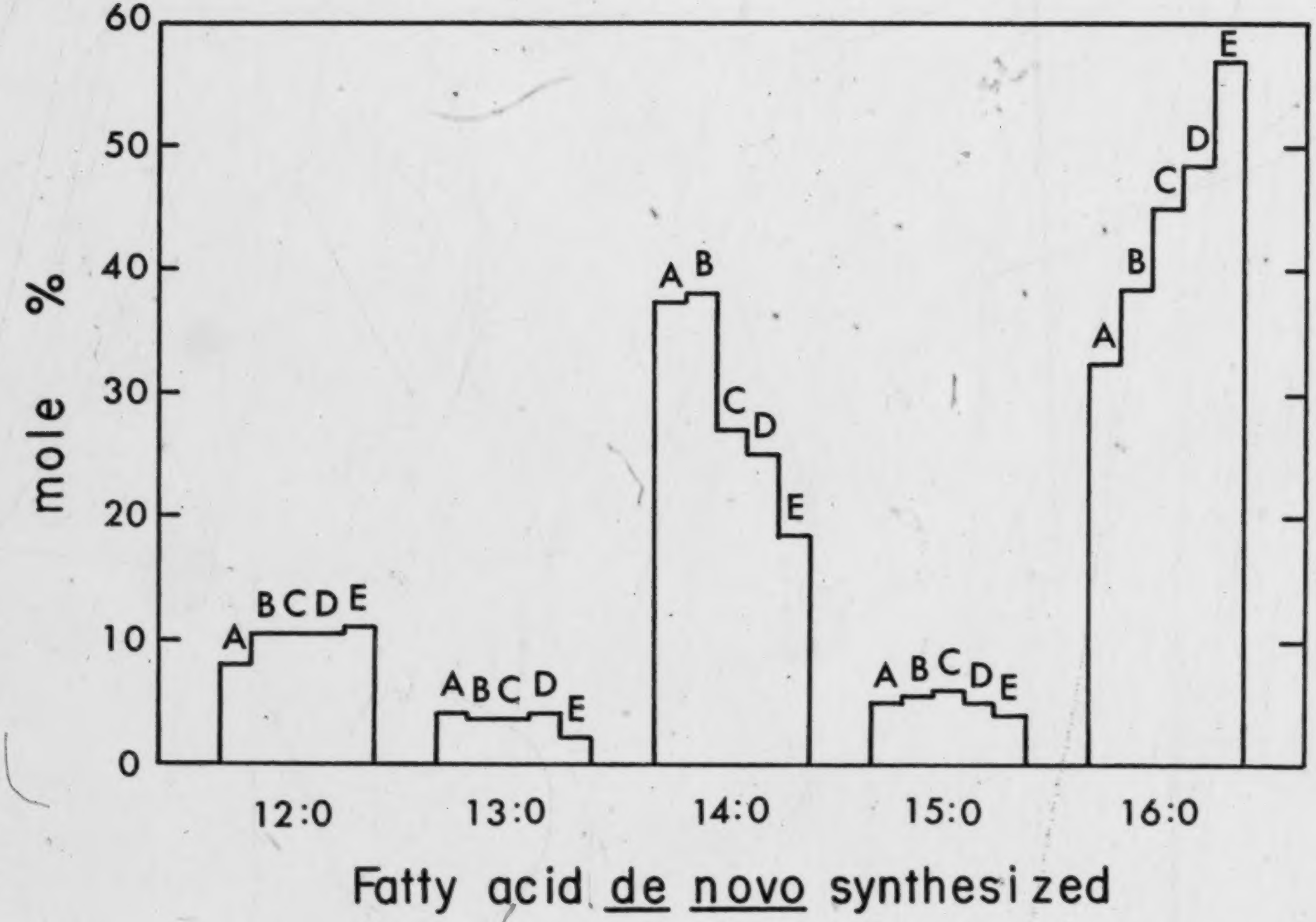
14:0
A B
C D
E
15:0
A B
C
D
E
16:0
Fatty acid de novo synthesized

amounts of 17:0. Increasing the amount of 17:0 incorporated increases the mole per cent of 12:0 and 13:0 present with a concomitant decrease in the levels of 16:0. The levels of 14:0 and 15:0 are not changed significantly. When 67.5 mole per cent of 17:0 is incorporated, 12:0 accounts for 31.7 mole per cent of the de novo synthesized fatty acids while 16:0 accounts for only 21.5 mole per cent. In the control experiment 12:0 and 16:0 account for 7.0 and 34.8 mole per cent of the total fatty acids, respectively. Figure 5 shows the effect of the increasing degree of incorporation of 9-18:1c. In contrast to 17:0, increasing the amount of 9-18:1c incorporated into the membrane lipids decreases the mole per cent of 14:0 biosynthesized with the concomitant increase in the levels of 16:0. When this organism incorporates up to 74.6 mole per cent of 9-18:1c, 14:0 and 16:0 occupy 19.3 and 62.1 mole per cent, respectively, of the fatty acid produced by the de novo pathway; in control experiments, 14:0 and 16:0 account for 36.7 and 34.8 mole per cent of the endogenous fatty acids, respectively.

As a more direct approach to this problem, use was made of sodium acetate-1,2-C^{14} as a precursor for de novo biosynthesis in the presence of one of the following exogenous fatty acids: 16:0, 17:0 and 9-18:1c. Since none of these exogenous fatty acids are significantly elongated, de novo synthesized acids alone are radiolabeled and detected by RAM. This method made it possible to use even 16:0 as an exogenous acid, because exogenous 16:0 does not interfere with the RAM reading of 16:0 derived from de novo biosynthesis. The experiment described in Figure 1 serves as a control, since it shows the pattern of fatty acids synthesized de novo in the absence of exogenous fatty acids. When *A. laidlawii* B incorporates 35.6 mole per cent of 17:0 into the

Fig. 5. The effect of the amount of exogenous 9-18:1c incorporated into membrane lipids on the composition of the fatty acid biosynthesized _de novo_ by _A_. _laidlawii_ B and incorporated into the membrane lipids. Cells were grown in the presence of varying amounts of 9-18:1c in 125 ml of the lipid-poor growth medium. Membrane lipids were extracted from late-log phase cells and methylesters of the membrane lipid fatty acids were prepared by the procedures described in the text. The fatty acid composition was analyzed by GLC, as described in the text; the relative amounts (expressed in mole per cent) of the various endogenous fatty acids were then calculated by ignoring the presence of any exogenous fatty acids. The amount of 9-18:1c incorporated into the membrane lipids in each case was: A, 0; B, 24.6; C, 42.8; D, 49.2; E, 74.6 mole per cent. All values presented here are representative of duplicate experiments.


mole %
60
50
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10
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A
B
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12:0
13:0
14:0
15:0
16:0
Fatty acid de novo synthesized

4

total membrane lipid, a greatly enhanced production of 12:0 and 13:0 and a dramatically reduced production of 16:0 are observed (Fig. 6). When this organism incorporates 53.4 mole per cent of 16:0 into the cell membrane, a very similar pattern is again observed. On the other hand, when 72.3 mole per cent of 9-18:1c is incorporated into the membrane lipids, 16:0 becomes the major fatty acid synthesized (Fig. 7). It is possible to calculate the composition of the endogenous fatty acids in the membrane lipids from these Figures simply by comparing the mole fractions of the fatty acids synthesized, which are obtained by dividing the area under RAM peaks by the number of radioactive carbon atoms in each molecule. The results are presented in Table 4. Although there are some minor differences in the pattern of *de novo* biosynthesis revealed by this technique, these results essentially confirm those obtained with the nonradioactive experiments described previously.

In order to fully evaluate the effect of fatty acid supplementation on *de novo* fatty acid biosynthesis, it is necessary to answer the following question: in the presence of exogenous fatty acids do cells reduce the *de novo* synthesis in proportion to their uptake of exogenous acids, or do they synthesize constant amounts and excrete excess products into the growth medium? To answer this question, lipids were extracted from the medium as well as from the cell membranes, which were separated by high-speed centrifugation. *De novo* synthesized fatty acids were analyzed by RAM-GLC. From the control experiment, where sodium acetate-1,2-C^{14} alone was added to the culture, no fatty acid with appreciable radioactivity is obtained from the medium. In the presence of 9-18:1c or 9-18:1t, again no fatty acids with

Fig. 6 . A RAM-GLC chromatogram illustrating the effect of the incorporation of exogenous 17:0 into membrane lipids on the composition of the fatty acids biosynthesized *de novo* by *A. laidlawii* B and incorporated into the membrane lipids. Cells were grown in the presence of both 1,2-C^{14}-acetate (6.25 x 10^{4}μC, 0.8μmoles) and 17:0 (1.2μmoles) in 10 ml of lipid-poor growth medium. The membrane lipids were extracted from late-log phase cells and methylesters were prepared by the procedures described in the text. The composition of the endogenous fatty acids was analyzed by RAM-GLC, as described in the text with 11:0 serving as an internal standard. Identification of the major peaks: A, solvent; B, 11.0; C, 12:0; D, 13:0; E, 14:0; F, 15:0; G, 16:0; H, 17:0; I, 18:0; J, 9-18:1c; K, 9,12-18:2c,c. - - - - - - - - and 〰〰 are the GLC (mass) and RAM (radioactivity) tracings respectively. This is a representative chromatogram from triplicate experiments.

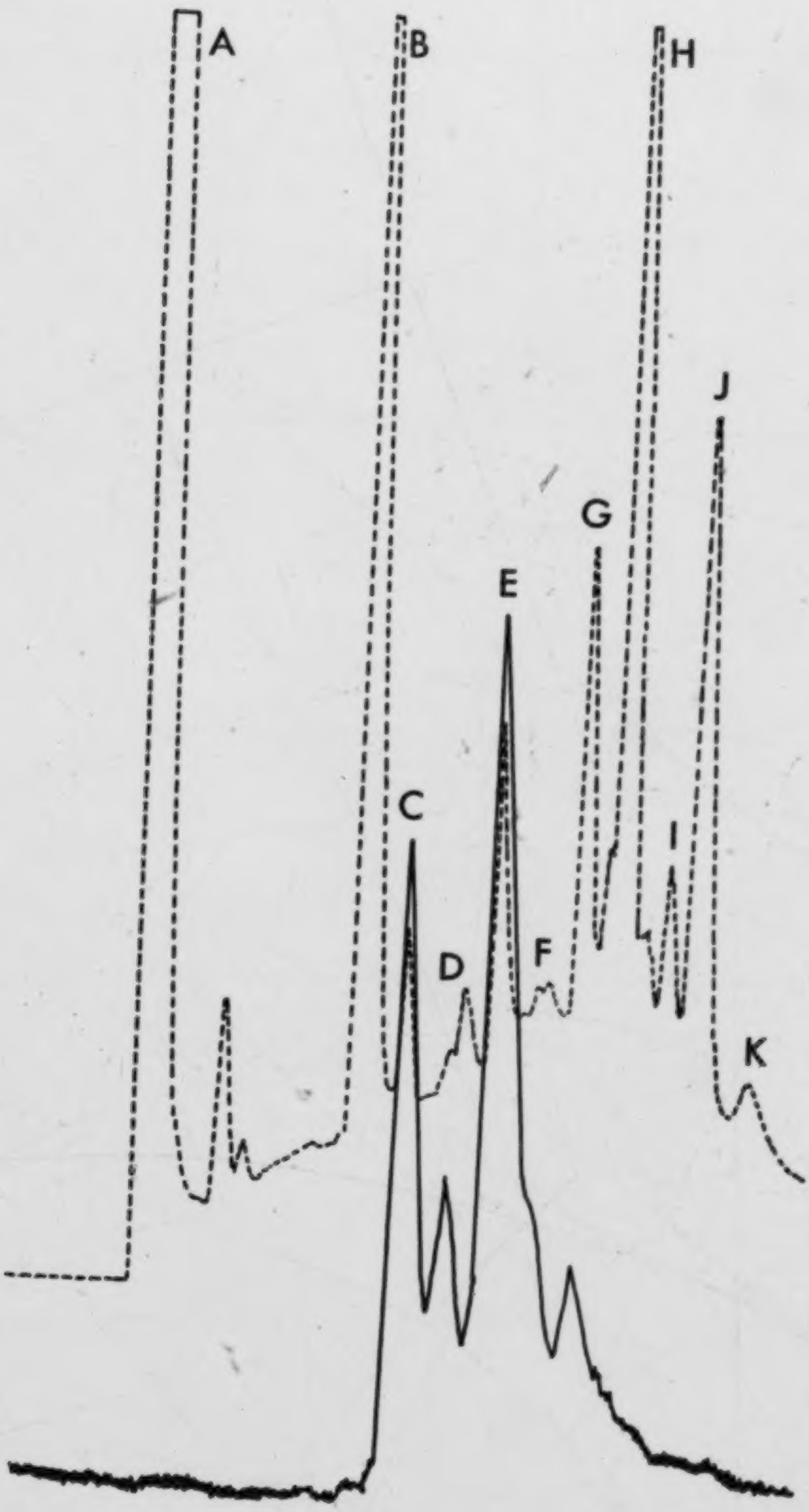
A
B
H
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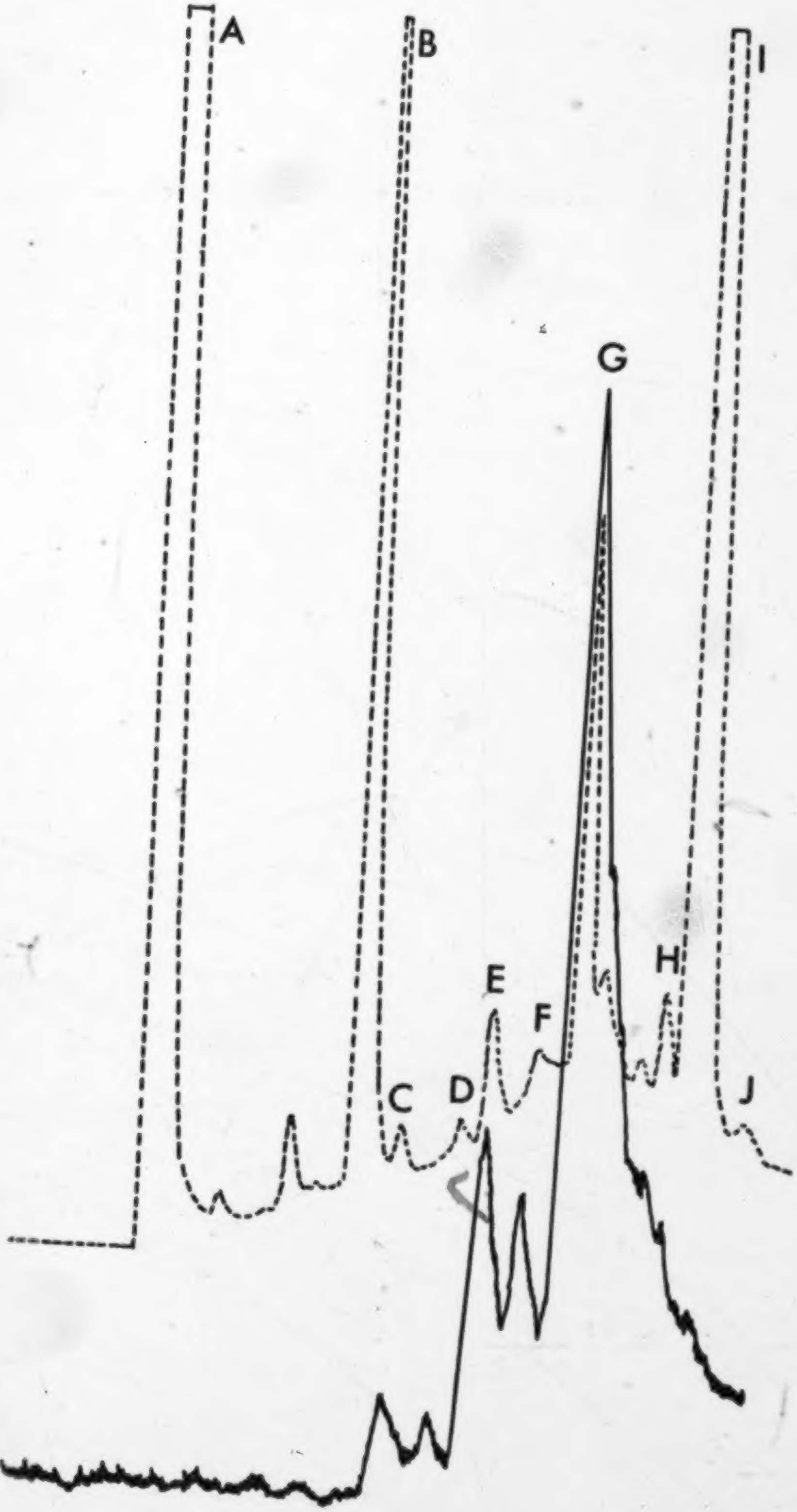
Fig. 7 . A RAM-GLC chromatogram illustrating the effect of the incorporation of exogenous 9-18:1c into membrane lipids on the composition of the fatty acids biosynthesized *de novo* by *A. laidlawii* B. Cells were grown in the presence of both 1,2-C^{14}-acetate (6.25 x 10^4 μC, 0.8μmole) and 9-18:1c (1.2μmoles) in 10 ml of lipid-poor growth medium. Membrane lipids were extracted from late-log phase cells, and methylesters were prepared by the procedures described in the text. The composition of the endogenous fatty acids was analyzed by RAM-GLC, as described in the text, with 11:0 serving as an internal standard. Identification of the major peaks: A, solvent; B, 11:0; C, 12:0; D, 13:0; E, 14:0; F, 15:0; G, 16:0; H, 18:0; I, 9-18:1c; J, 9,12-18:2c,c. -------- and —•— are GLC (mass) and RAM (radioactivity) tracings, respectively. This is a representative chromatogram from triplicate experiments.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significant radioactivity were detected in the growth medium. On the other hand, in the presence of 17:0 (or 16.0), substantial amounts of radioactive long-chain saturated fatty acids are detected in the growth medium, as is illustrated in Fig. 8 . Using the same methodology previously utilized for the cell membrane, the relative quantities of each de novo biosynthesized fatty acid found in the growth medium was calculated, and the results are presented in Table 5. A comparison of Tables 4 and 5 reveals that the spectra of fatty acids present in the total membrane lipids and in the growth medium are markedly different. In the membrane lipids 14:0 is the major endogenous fatty acid, while in the growth medium 12:0 is the predominant fatty acid derived from the de novo pathway. Thus, fatty acids are not released into the growth medium in a random fashion where cells are growing in the presence of certain long-chain saturated fatty acids. It is also of interest to note that although the patterns of endogenous fatty acids found in the total membrane lipids from cells supplemented with 16:0 or 17:0 are almost identical (Table 4), the composition of de novo biosynthesized fatty acids found in the growth medium of cells supplemented with these two fatty acids are somewhat different (Table 5).

Since 11:0 was added as an internal standard to the lipid extracts from both cell membranes and growth medium it is possible to calculate directly the proportion of the total fatty acid biosynthesized which is present in the cell membrane and in the growth medium. The results of this calculation are presented in Table 6. It is clearly

Fig. 8 . A RAM-GLC chromatogram illustrating the effect of exogenous 17:0 on the excretion of fatty acids biosynthesized *de novo* by *A. laidlawii* B into the growth medium. Cells were grown in the presence of both 1,2-C^{14}-acetate (6.25 x 10^4µC; 0.8µmole) and 17:0 (1.2µmoles) in 10 ml of the lipid-poor growth medium. The medium was separated from the late-log phase cells by high-speed centrifugation as described in the text. The lipids were extracted from the resultant cell-free medium, and methylesters were prepared by the procedures described in the text. The composition of the excreted endogenous fatty acids was analyzed by RAM-GLC, as described in the text, with 11:0 serving as an internal standard. Identification of the major peaks: A, solvent; B, 11:0; C, 12:0; D, 13:0; E, 14:0; F, 15:0; G, 16:0; H, 17:0; I, 18:0; J, 9-18:1c; K, 9,12-18:2c,c. --------- and ——— are the GLC (mass) and RAM (radioactivity) tracings, respectively. This is a representative chromatogram from duplicate experiments.

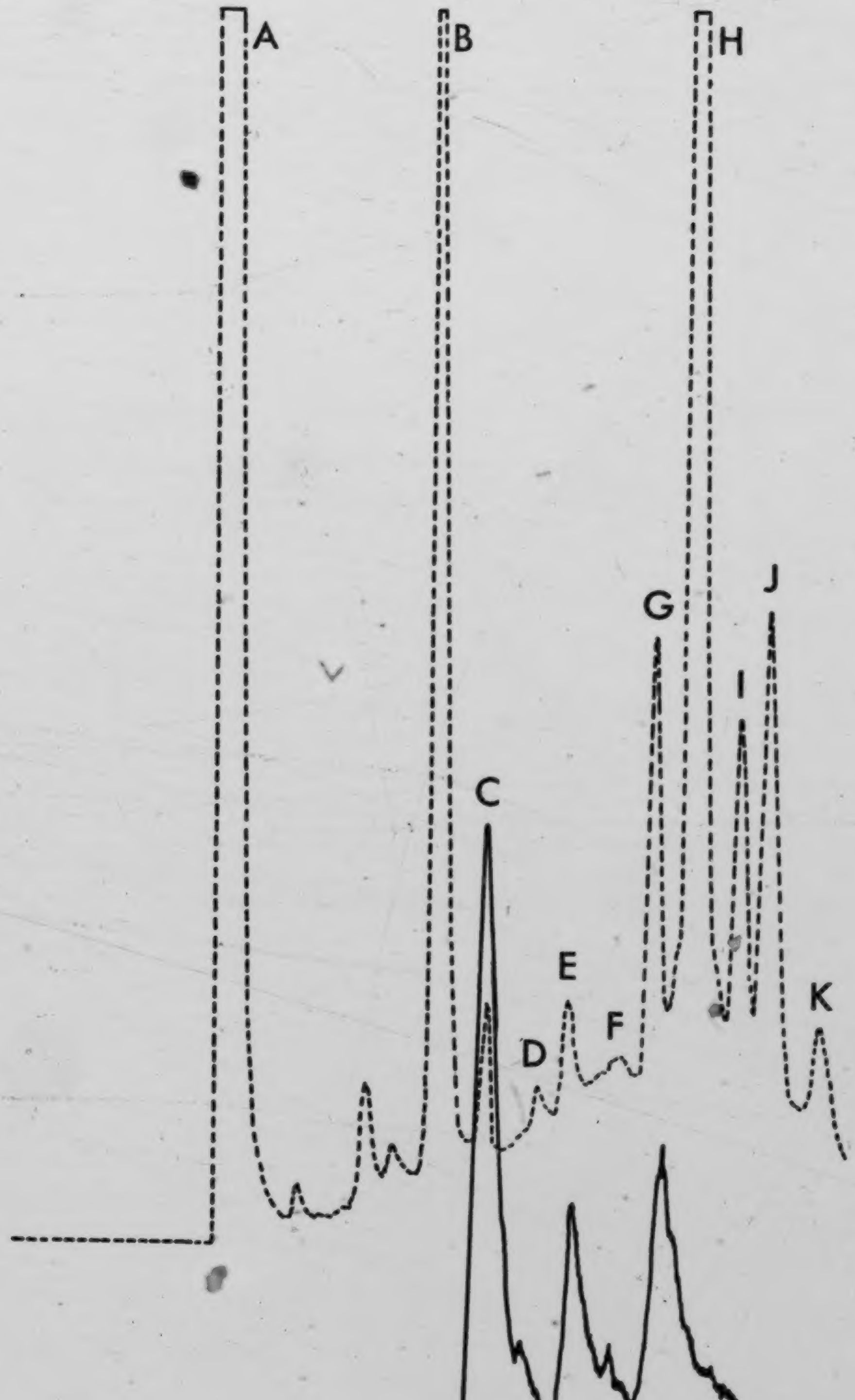
A
B
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Table 5. The effect of exogenous saturated fatty acids on the composition of the fatty acids synthesized de novo by A. laidlawii B and excreted into the growth medium

Endogenous fatty acids found	Exogenous fatty acids[a]	
	17:0	16:0
12:0	48.2[b]	40.0
13:0	6.7	-
14:0	19.8	27.6
16:0	25.4	32.5

[a] The amounts of the exogenous 17:0 and 16:0 incorporated into the total membrane lipids are 35.6 and 53.4 mole per cent, respectively.

[b] All fatty acid compositional values are expressed in mole per cent and are obtained from representative RAM-GLC chromatograms.

Table 6. The effect of various exogenous fatty acids on the distribution of fatty acids synthesized de novo by A. laidlawii B between the cell membrane and the growth medium

Exogenous fatty acid[a]	Cell membrane (%)	Growth medium (%)
none	94.9	5.1
9-18:1c	95.0	5.0
17:0	57.7	42.3
16:0	76.0	24.0

[a] The amounts of the various exogenous fatty acids incorporated into the membrane lipids are given in Table 4. The endogenous fatty acid distributional values shown here are the representative results of at least duplicate experiments.

shown that cells grown in the presence of acetate alone, or acetate plus 9-18:1c, do not excrete de novo biosynthesized fatty acids into the growth medium to any appreciable extent. On the other hand, cells grown with acetate plus 16:0 or 17:0 excrete substantial amounts of endogenous fatty acids into the growth medium (24.0 and 42.3 per cent respectively).

If we assume that the total amounts of fatty acid (the number of molecules) present in the membrane of cells grown in the presence of various exogenous acids, or without fatty acid supplementation, is constant, we can roughly calculate the relative amounts of the total fatty acid produced by the de novo pathway, present in the membrane by the following equation:

$$\text{The relative amounts of de novo synthesized total fatty acids found in the membrane} = \frac{\text{Total area under RAM peaks in the membrane}}{\text{Total mole fractions of fatty acids in the membrane}} \times 100$$

Since we already calculated the proportion of the de novo synthesized fatty acids found in the membrane, compared to the total amount biosynthesized, when cells were grown in the absence of exogenous fatty acids or in the presence of 9-18:1c, 17:0 or 16:0 (Table 6), we can also calculate the total amount of the de novo biosynthesized fatty acid produced by the cells in each case by measuring the relative amount of endogenous fatty acid present in the cell membrane. The results of these calculations are presented in Table 7. By comparing the relative amount of the total de novo biosynthesized

Table 7 . The effect of various exogenous fatty acids on the relative amounts (expressed in arbitrary units) of fatty acid synthesized *de novo* by *A. laidlawii* B cells

Exogenous fatty acid[a]	Amount of endogenous fatty acid in the membrane	Total amount endogenous fatty acid synthesized by the cell
none	25.40	26.77[b]
9-18:1c	11.41	12.02
17:0	13.38	23.20
16:0	11.48	15.11

[a] The amounts of the various exogenous fatty acids incorporated into the total membrane lipids are given in Table 4.

[b] The values shown here are the representative results of at least duplicate experiments.

A. laidlawii B, which are inoculated in 100 ml of growth medium, synthesize about 2.3 mg of fatty acids in 24 hrs. in the absence of exogenous fatty acids. If the amount of fatty acyl moiety in the cell membrane is fairly constant, cells in 100 ml of growth medium synthesize 2.0 and 1.3 mg and excrete 0.9 mg and 0.3 mg of fatty acids in the presence of 17:0 and 16:0, respectively.

fatty acids found in the membrane in the presence of an exogenous fatty acid to the relative amount found in the absence of exogenous fatty acid, the effectiveness of 9-18:1c, 17:0 and 16:0 in displacing endogenous fatty acids from the membrane lipids can be determined. For example, in the presence of 9-18:1c the relative amount of _de novo_ biosynthesized fatty acids found in the membrane is 11.41, compared to the control value of 25.40. The extent of displacement of the endogenous fatty acids is calculated as 100 (25.40 - 11.41)/25.40 = 55.1%. On the other hand, a comparison of the relative amount of the total fatty acid biosynthesized _de novo_ by the cells in the presence of exogenous fatty acid to the relative amount biosynthesized in the absence of exogenous fatty acid, would give the effectiveness of the exogenous acid in inhibiting fatty acid biosynthesis. For example, in the presence of 9-18:1c the relative amount of _de novo_ biosynthesized total fatty acids is 12.02 compared to the control value of 26.77. The extent of inhibition of _de novo_ fatty acid biosynthesis is calculated as 100 (26.77 - 12.02)/26.77 = 55.1%. The results of these calculations are presented in Table 8 , which also includes the amount (mole per cent) of the exogenous fatty acids incorporated into the total membrane lipid, after being corrected for the amount of 9-18:1c found in each experiment to be derived from residual 9-18:1c in the growth medium. When 9-18:1c is present in the growth medium, this unsaturated fatty acid accounts for 54.3 mole per cent of the total esterified fatty acid in the membrane lipid. The total biosynthesis of endogenous fatty acids, Table 8 . The effect of various exogenous fatty acids on the extent of displacement of endogenous fatty acids from the cell membrane and on the extent of inhibition of de novo fatty acid biosynthesis by A. laidlawii B

Exogenous fatty acid	Displacement[a] (%)	Inhibition[b] (%)	Incorporation[c] (mole %)
9-18:1c	55.1	55.1	54.3
17:0	47.3	13.3	40.6
16:0	54.8	43.6	53.4

[a] The extent of displacement of endogenous fatty acids from the cell membrane is defined as the percentage reduction of the total esterified fatty acid which is derived from de novo biosynthesis in cells grown in the presence of an exogenous fatty acid relative to cells grown without fatty acid supplementation.

[b] The extent of inhibition of de novo fatty acid biosynthesis is defined as the percentage reduction in the total amount of endogenous fatty acid present in both the cell membrane and the growth medium observed in the presence of an exogenous fatty acid.

[c] The extent of incorporation is defined as the amount of exogenous fatty acid incorporated into the total membrane lipids.

The values shown here are the representative results of at least duplicate experiments.

as measured by the production of radioactive long-chain fatty acids from ^{14}C-acetate, is found to be reduced by 55.1 per cent in the presence of 18:1c as compared to control cells. Under these conditions almost no endogenous fatty acids are released to the growth medium, so 18:1c appears to reduce *de novo* fatty acid biosynthesis by a direct inhibition of the fatty acid synthetase complex, and not at all by a displacement of endogenous fatty acids from the cellular lipids into the growth medium. On the other hand, the incorporation of 53.4 and 40.6 mole per cent of exogenous 16:0 and 17:0, respectively, into the plasma membrane reduces the amount of exogenous fatty acid *in the membrane* by 54.8 and 47.3 per cent. However, the inhibition of total fatty acid *de novo* biosynthesis for the plasma membrane and growth medium combined is only 43.6 per cent for 16:0 and just 13.3 per cent for 17:0. Thus these long-chain saturated fatty acids are not as potent as 18:1c in reducing the total output of the *de novo* fatty acid synthetase complex, but appear to function instead by displacing endogenous fatty acids, which continue to be produced in amounts greater than apparently required for membrane lipid biosynthesis, into the growth medium.

In summary, if one can accept the reasonable assumption that the total quantity of endogenous plus exogenous fatty acid in cells remains relatively constant, then these results suggest that under certain circumstances the *de novo* fatty acid biosynthetic pathway in *A. laidlawii* B is subject to stringent regulation by the presence of an unsaturated fatty acid in the growth medium. Under other circumstances, the inhibition of fatty acid biosynthesis by certain exogenous saturated fatty acids is not tightly coupled to the incorporation of these fatty acid supplements, since endogenous fatty acids continue to be synthesized but are excreted into the growth medium, instead of being totally incorporated into the membrane polar lipids.

3. Discussion

Differential calorimetrical studies performed on A. laidlawii B membranes and membrane lipids have provided evidence for the "bilayer" structure of lipids in the cell membrane of this organism (Steim *et al.*, 1969). Reversible, thermotropic transitions from the gel to liquid-crystalline state, which arise from a cooperative melting of the hydrocarbon chains of the membrane lipids, were detected in the membranes of this organism. Phase transitions, with similar properties and which occurred over the same temperature range, could also be domonstrated in the total lipid extracts of these cells, when the extracted lipid was dispersed as bilayer lamallae in excess water. The incorporation of increasing amounts of cholesterol into the plasma membrane broadened and eventually abolished the gel to liquid-crystalline phase transition by destroying the cooperativity of chain-melting. About 90 per cent of the lipids in the membrane appeared to be involved in the phase change. These results suggest that the lipids in the membrane of A. laidlawii B exist primarily in bilayer structure in which lipid-lipid non-polar interactions, as opposed to lipid-protein interactions, are of major importance. The existence of extensive domains of lipid bilayer structure was further substantiated by studies utilizing other physical methods, including X-ray diffraction (Engelman, 1970, 1971), and electron paramagnetic resonance spectroscopy

(Tourtellotte et al., 1970). The forces which stabilize the lipid bilayer structure are mainly van der Waals interactions between adjacent fatty acyl hydrocarbon chains in the apolar core of the bilayer. The number of methylene groups and the chemical nature of fatty acyl groups in the membrane lipids would, therefore, have profound effects on these stabilizing forces, and on the physical state of the membranes. Studies with synthetic phospholipids have revealed that shortening the chain length or increasing the degree of unsaturation in the fatty acyl residues results in increasing the fluidity of the lipid films, and lowering the temperatures at which gel to liquid-crystalline phase changes are observed (Chapman et al., 1967). Biomembranes are known to perform various biologically important functions. Many studies have shown that the phase state of the membrane lipids can have a profound effect on a variety of membrane-associated enzyme and transport functions (McElhaney et al., 1970, 1973; Romijn et al., 1972; Van Deenen, 1972; Kimelberg and Papahadjopoulos, 1974).

A. laidlawii B is one of the relatively few microorganisms thus far discovered which can tolerate profound alterations in the fatty acid composition of its membrane lipids (McElhaney and Tourtellotte, 1969). Despite this ability to tolerate relatively large changes in the physical state of the membrane lipid, this organism does seem to possess certain control mechanisms which act to maintain the fluidity of the membrane lipids within certain limits. The ability to alter in a characteristic way the average chain length of the fatty acids produced by the de novo biosynthetic pathway in response to the presence of exogenous fatty acids would appear to be one such mechanism. Others will be discussed elsewhere in this dissertation. By altering the physical properties of the endogenous fatty acids, A. laidlawii B can apparently cushion some of the adverse effects of the incorporation of certain exogenous fatty acids on membrane lipid fluidity.

The experiments discussed in this section of the thesis indicate clearly that the de novo fatty acid biosynthetic system can make appropriate alterations in the spectrum of the fatty acids produced in response to the physical properties of the exogenous fatty acid. Where the exogenous fatty acid is a high-melting, long-chain saturated fatty acid, the de novo fatty acid synthesis pattern is changed to produce preferentially short-chain acids. In the case where the exogenous acid is a lower-melting cis-monounsaturated acid, longer-chain fatty acids are preferentially synthesized. Similar results are also observed when other low-melting cis-monounsaturated, polyunsaturated, and cyclopropane ring-containing fatty acids are added to the growth medium. These results can be interpreted as a successful attempt by the cells to compensate for the effects of exogenously added fatty acids on the fluidity of the membrane lipid. In the absence of exogenous fatty acids A. laidlawii B biosynthesizes roughly equal amounts of 14:0 and 16:0 (about 30 to 40 mole per cent each) and smaller amounts of 12:0, 13:0 and 15:0 (5 to 10 mole per cent each). The incorporation into the membrane lipid of 17:0, which has more methylene groups than any de novo synthesized fatty acid, enhances the cohesive attractive forces among fatty acyl hydrocarbon chains and decreases the fluidity of the membrane by increasing the strength of van der Waals interactions. On the other hand, the incorporation of 9-18:1c, a *cis*-monounsaturated fatty acid, would greatly reduce the cohesive attractive forces among fatty acyl hydrocarbon chains and increase the fluidity of the membranes, because the *cis*-double bond creates a "kink" in the hydrocarbon chains, which disrupts the close packing of fatty acyl chains, thereby decreasing both van der Waals and hydrophobic interactions (Van Deenen, 1962). The incorporation of higher-melting exogenous acid is compensated for by the increased *de novo* biosynthesis of fatty acids (12:0 and 13:0), which tend to reduce the melting point or phase transition temperature. The incorporation of lower melting fatty acid results in the preferential *de novo* synthesis of the longer-chain endogenous fatty acids. This compensatory effect is not confined to *A. laidlawii* B; it has been observed in bacteria, yeast, and eukaryotic cells as well.

Escherichia coli is another organism whose fatty acid metabolism has been very extensively studied, and which has been successfully used to elucidate the structure and function of fatty acyl residues in biomembranes (Esfahani *et al.*, 1971a; Tsukagoshi and Fox, 1973; Linden *et al.*, 1973). Wild type *E. coli* synthesizes about 50 to 60 per cent unsaturated fatty acids and incorporates these monounsaturates into its membrane lipids (Silbert *et al.*, 1968). *E. coli* K-12 was shown to be capable of regulating the ratio of unsaturated to saturated fatty acid biosynthesis in response to saturated and/or unsaturated fatty acid supplements in the medium, so as to maintain the fluidity of the membrane lipids within a certain range (Sinensky, 1971; Silbert *et al.*, 1972, 1973a). Mutants of *E. coli* which require unsaturated fatty acids for growth were discovered. Some of them lacked a *beta*-hydroxydecanoyl thioester dehydrase (Silbert and Vagelos, 1967; Cronan, et al., 1969, 1972), which is a key enzyme in the anaerobic biosynthesis of unsaturated fatty acids (Norris et al., 1964; Brock et al., 1966; Kass and Bloch, 1967). These mutants have been used very successfully, because their fatty acid composition can be more systematically altered than the wild type strains. E. coli unsaturated fatty acid auxotrophs were pre-grown in media supplemented with 9-18:1c. The culture was harvested, washed, and resuspended in fresh media to give two separate cultures, only one of which contained 9-18:1c. The supplemented and deprived cultures were again incubated for one more generation in the presence of C^{14}-acetate. The overall fatty acid composition was shifted from 56 per cent saturated and 44 per cent unsaturated fatty acids to 82 per cent saturated and 18 per cent unsaturated acids in the phosphatidylethanolamine of the starved cells. This change in saturated acids was due to a very large incorporation of 14:0 over 16:0, the latter being by far the major saturated acid synthesized in the supplemented cells (Silbert, 1970). This increased production of the shorter-chain saturated acids by the starvation of unsaturated fatty acid can be interpreted as a compensatory attempt by the organism to maintain the membrane lipid fluidity within a certain range. E. coli double mutants, which lack beta-oxidation as well as unsaturated fatty acid biosynthesis (similar to A. laidlawii B in terms of fatty acid metabolism) were grown in the presence of various unsaturated fatty acids, and the fatty acid compositions of phospholipids were analyzed (Esfahani et al., 1971a). When supplemented with 18:1, the ratios of 16:0 to 14:0 were always higher in cells supplemented with cis-acids, as compared to those supplemented with the corresponding trans-acids, and the 16:0 to 14:0 ratios were always higher in cells supplemented with monounsaturates having a double bond at position 9 as compared to those having a double bond at position 11 if the geometrical configurations of the double bonds were the same. When supplemented with 11-20:1c series, increasing the extent of unsaturation increased gradually the ratio of 16:0 to 14:0. These results can also be interpreted as successful compensatory effects by the organism to keep the membrane lipid fluidity within a certain range. It has been shown that similar mechanisms are also operative in higher organisms. Meyer and Bloch (1963) found that in anaerobically-grown yeast, the decrease of unsaturated fatty acids was compensated for by an appearance of saturated fatty acids of shorter-chain length. Van Golde and Van Deenen (1966) have shown that the effect of variations in membrane fatty acids of rat liver due to dietary alterations may be compensated for by the biosynthesis of fatty acids with related degrees of unsaturation.

The excretion of de novo synthesized fatty acids into growth medium in the presence of certain exogenous saturated fatty acids is a rather unexpected finding. There is very little excretion of de novo synthesized fatty acids in the control experiment, where acetate alone is added to the medium, and when either 9-18:1c or 9-18:1t is added to the medium. When 17:0 or 16:0 is added, very substantial amounts of de novo synthesized fatty acids are excreted, 42.3 and 24.0 per cent, respectively. Why some exogenous fatty acids produce excretion of endogenous fatty acids and some do not is not clearly understood. Whether the excreted radioactive fatty acids are free fatty acids or components of some complex lipid is not yet known. Some strains of E. coli and Salmonella typhimurium (Knox et al., 1966, 1967; Rothfield and Pearlman-Kothencz, 1969; Silbert et al., 1972) are known to excrete fatty acids in the form of lipopolysaccharide-phospholipid-protein complexes. Rothfield and Pearlman-Kothencz (1969) indicated that membrane complexes were continually excreted, presumably from the outer membrane (Costerton et al., 1974) by normally growing bacteria and that increased excretion of the complexes results from cessation of protein synthesis, possibly to compensate for the unbalanced production between proteins and lipids. Some exogenous fatty acids, like 9-18:1t, seem able to inhibit the de novo fatty acid synthetase so as to make the amount of total fatty acids in the membrane, including the acids incorporated from the medium, constant; some, like 16:0 or 17:0, may not.

Although some organisms can tolerate a substantial imbalance between membrane lipids and proteins under some special conditions (Mindichi, 1970; Henry, 1973), it would be reasonable to assume that the amount of fatty acyl chains in membranes of most cells might be fairly constant. Cells utilizing exogenous fatty acids, then, must inhibit the de novo synthesis of fatty acids so as to maintain the total amount of membrane fatty acyl chains constant. Otherwise, the cells must excrete some excess fatty acids (or complex lipids) into the medium. With regard to the above speculation it is worth mentioning the study of Lactobacilli. Hofmann et al. (1959) found that L. arabinosus and L. casei failed to produce measurable amounts of cis-vaccenic acid when lactobacillic acid replaced biotin in their culture medium. Henderson and McNeil (1966, 1967) found that exogenous monounsaturated and cyclopropanoid fatty acids, but not saturated acids, inhibited the long-chain fatty acid synthesis in L. plantarum, presumably by the end-product inhibition of acetyl-CoA carboxylase. Birnbaum (1970) suggested the repression of acetyl-CoA carboxylase synthesis by long-chain unsaturated fatty acids as the mechanism of the inhibition, rather than the end-product inhibition on the enzyme activity. Weeks and Wakil (1970) found that the synthesis of several components of the fatty acid synthetase complex, as well as the synthesis of acetyl-CoA carboxylase, were repressed in L. plantarum when they were grown with monounsaturated acids. They also found a decreased amount of Acyl Carrier Protein (ACP) in the cells grown with unsaturated acids. Evidence for feedback inhibition has never been obtained in this system. No evidence is currently available to indicate whether the repressor substance is the free fatty acid, the fatty acyl-CoA, the fatty acyl-ACP, or some other fatty acyl derivative. In L. plantarum the major de novo synthesized fatty acids are 11-18:1c, cis-11,12-methylene octadecanoic acid (11,12-19:0 cp) and 16:0, whereas A. laidlawii B synthesizes strictly saturated acids. This would suggest that the same control mechanism present in L. plantarum might not be operative in A. laidlawii B. Because it is more natural to assume that the normal products of synthesis (saturated acids), not non-products (unsaturated acids), would inhibit the biosynthetic pathway more effectively. Studies designed to elucidate the mechanism by which various exogenous fatty acids inhibit de novo biosynthesis in this organism are currently underway.

C. The effect of variations in growth temperature and membrane cholesterol content on fatty acid de novo biosynthesis

1. Materials and Methods

The effect of cholesterol: A sterile ethanolic solution of cholesterol (10 mg/ml) was added to 125 ml of lipid-poor growth medium before inoculation to bring the final concentration of cholesterol in the medium to 25 mg/l. Cholesterol was ULTREX grade from Baker Chemical Company (Phillipsburg, New Jersey, U.S.A.). Cells were harvested in late-log to early-stationary phase, after about 16 hours of growth. Lipids were extracted, methyl esters of fatty acids were prepared, and the fatty acid composition was determined as described previously.

The effect of growth temperature: Cells were grown in 250 ml lipid-poor media at 2°C intervals from 4° to 48°C. The cell growth was followed by the absorbancy at 450 mm. As soon as cell growth ceased, the cells were collected, lipids were extracted, methyl esters of fatty acids were prepared, and the fatty acid composition was determined as described previously.

2. Results

Although the morphology of the cells is changed to somewhat more coccoidal shape when grown in the presence of cholesterol, the fatty acid composition of the total membrane lipids is identical within an experimental error to the control culture, which was grown in the absence of cholesterol, as can be seen in Table 9. The growth temperature affects only moderately the fatty acid composition of the

Table 9. The effect of cholesterol on the fatty acid composition of the total membrane lipids of A. laidlawii B grown without exogenous fatty acids

Fatty acids found	Without cholesterol (mole %)	With cholesterol (mole %)
12:0	6.6[a]	6.4[a]
13:0	2.3	2.5
14:0	33.4	31.9
15:0	4.3	4.9
16:0	42.0	42.8
17:0	1.1	1.2
18:0	5.5	5.6
9-18:1c	4.8	4.7

[a] These fatty acid compositional values are the averages of duplicate experiments.

total membrane lipids, which is shown in Table 10.

3. Discussion

A. laidlawii B does not biosynthesize cholesterol or require cholesterol for growth. Thus, when grown without cholesterol supplementation, the membrane of this organism does not contain cholesterol. However, when grown with cholesterol, *A. laidlawii* B can incorporate this sterol into its membrane lipid bilayer in a chemically unmodified form. At the concentration of the sterol used, 25 mg/l culture, this organism is known to incorporate the sterol to a maximum level of about 8 weight per cent of total lipids, which is about 18 mole per cent of total lipids, assuming an average molecular weight of lipid in this organism of 870 (De Kruyff *et al.*, 1972).

Monolayer studies on the interaction of phospholipids and cholesterol (Demel *et al.*, 1967) demonstrate that cholesterol can interact with certain phospholipids and reduce the mean area per molecule in the monolayer film (condensing effect). It was shown by electron spin resonance spectroscopy studies (Oldfield and Chapman, 1971) that cholesterol interacted not only with lipids which were in liquid-crystalline state to reduce the mobility of the fatty acyl chains, but also with gel lipids to increase the mobility of the fatty acyl chains (liquefying effect). Calorimetric studies (Ladbrooke *et al.*, 1968) revealed that the interaction of cholesterol with lipids in either the gel or liquid-crystalline state decreased the heat absorbed at the gel to liquid-crystalline phase transitions.

Differential scanning calorimetry studies (De Kruyff *et al.*, 1973) showed that when cholesterol is mixed with a codispersion of

Table 10. The Fatty acid composition of the total membrane lipids of A. laidlawii B grown without fatty acid supplementation at various temperatures

Fatty acids found	Temperature of growth (^{o}C)				
	40	35	30	25	20
12:0	4.8[a]	5.0	5.9	8.2	9.6
13:0	3.4	3.4	4.8	6.2	6.5
14:0	30.2	30.8	30.0	28.4	30.1
15:0	7.7	6.6	8.1	8.1	7.2
16:0	40.8	40.1	39.0	37.5	36.9
17:0	2.1	3.2	2.4	1.9	1.2
18:0	8.0	7.7	7.3	7.1	6.5
18:1	1.4	1.8	1.2	1.5	1.2
18:2	1.5	1.2	1.2	1.0	0.8

[a] All fatty acid compositional values are expressed as mole per cent.

liquid-crystalline lipid (dioleoylphosphorylcholine), and crystalline lipid (distearoylphosphorylcholine) (1.35:1 mixture), it bound specifically with the former lipid to condense its hydrocarbon core, providing the concentration of cholesterol was less than 23.3 mole per cent. McElhaney (1974) studied the thermal behavior of the membrane lipids of A. laidlawii and concluded that at the growth temperature, 34°C, lipids in the membrane from the cells grown without fatty acid supplementations existed as roughly equimolar mixtures of liquid-crystalline and gel states.

It is, therefore, reasonable to assume that cholesterol incorporated (less than 20 mole per cent) in A. laidlawii B membrane lipids, which are codispersion of liquid-crystalline and crystalline lipids, binds preferentially with the liquid-crystalline region of the membrane lipids and reduces the fluidity and mobility of the hydrocarbon core. In analogy to the effect of exogenous saturated long-chain fatty acids, de novo synthesis is expected to compensate for the "condensing" effect of cholesterol by decreasing the average chain length. However this is not observed. The cell must respond to the effect of exogenous fatty acids not simply by their hydrophobic interactions with membrane lipids, but by some more specific interaction.

The permissible temperature range for cell growth is 20° to 44°C when cells are grown without any fatty acid supplementation (McElhaney, 1974). Within this temperature range the fatty acid composition of the total membrane lipids is only moderately affected; the synthesis of the shorter-chain fatty acids (12:0 and 13:0) is slightly increased, and that of the longer-chain fatty acids (16:0, 17:0 and 18:0) is somewhat reduced at lower growth temperatures. This organism does not seem to have a highly effective control mechanism to change the fluidity of membrane lipids by altering the de novo fatty acid synthetic pattern in response to changes in the environmental temperature. In fact, the maximum cell density as well as the growth rate were highly dependent on the growth temperature (McElhaney, 1974). The very closely related organism, A. laidlawii A, which requires unsaturated fatty acid for growth, was also reported not to change its fatty acid composition when grown at 37° and 25°C (Rottem and Panos, 1969).

Many reports have been made about the effect of the growth temperature on the de novo synthetic fatty acid pattern in both prokaryotic and eukaryotic organisms. For example, psychrophilic microorganisms generally have more highly unsaturated fatty acids than their mesophilic counterparts, and thermophilic microorganisms generally have more highly saturated fatty acids than mesophiles (Summer and Morgan, 1969). Furthermore, when the same organism is grown at two different temperatures it usually synthesizes more unsaturated fatty acids at the lower temperature, and more saturated fatty acids at the higher temperature. Isobranched fatty acids are often synthesized in larger amounts at higher temperatures; anteisobranched acids are typically synthesized in decreasing quantities at higher temperatures. These changes usually can be interpreted as a physiological adaptive response to changing environmental temperature, which function to counteract the effect of the temperature change on the fluidity of the membrane lipid. Recently Sinensky (1974) has introduced the term "Homeoviscous adaptation". The fluidity of the membrane and the extracted lipids from E. coli K-12 cells grown at various temperatures was determined by electron spin resonsnce spectroscopy at the temperature of growth. He found that the viscosity or fluidity of the hydrocarbon core of the membrane lipids was very constant as long as the measurement was done at the temperature of growth. This experiment has clearly demonstrated that the cells change the fatty acid composition in response to the change of the growth temperature, so as to maintain the membrane lipid fluidity constant regardless of the growth temperature. However, the mechanism or mechanisms involved in this response seem to be quite complex, and only a few reports have been published which deal with possible molecular mechanisms for the regulation of membrane lipid fluidity.

A dramatic effect of growth temperature on the fatty acid composition of E. coli has been reported by Marr and Ingraham (1962). Lowering the growth temperature enhances the synthesis of monounsaturated fatty acids and reduces the production of saturated acids. Sinensky (1971) studied the effect of temperature on the incorporation of 16:0 and 9-18:1c in vivo as well as in vitro, when these two fatty acids constituted about 90 per cent of the total fatty acids. From these studies he concluded that the acyl-CoA:glycero-3-phosphate transferase was the enzyme responsible for changing the fatty acid composition in phospholipids, in response to variations in the temperature of growth. He also concluded that the specificity of this enzyme, but not the synthesis of the enzyme, was changed in response to the change of the environmental temperature. Whether this mechanism is operative for the *de novo* synthesized fatty acids, as well as for exogenously added fatty acids, remains to be determined.

Bacillus licheniformis also increases the production of unsaturated fatty acids at lower growth temperatures. Unlike *E. coli*, bacilli synthesize unsaturated fatty acids by the direct oxidative desaturation of saturated acid precursors. One of the two desaturases present in this organism functions only at low temperatures, apparently due to the cold-induction of the enzyme as well as to the decreased inactivation of the enzyme itself and the enzyme synthesizing system at lower temperatures (Fulco, 1970, 1972).

Generally speaking, organisms respond to a change of growth temperature mainly by changing the ratio between the saturated and the unsaturated fatty acids in the membrane lipid rather than by changing the chain length. *A. laidlawii* B does not synthesize any unsaturated fatty acids, and can not respond efficiently to the change of the growth temperature. *Staphylococcus aureus* does not synthesize appreciable amounts of unsaturated fatty acids, and also can not change fatty acid composition when grown at 37 and 25°C (Joyce *et al*., 1970).

CHAPTER II

THE INCORPORATION OF EXOGENOUS FATTY ACIDS INTO THE MEMBRANE LIPIDS

Introduction

A number of microorganisms are capable of taking up exogenous fatty acids from the growth medium and of incorporating such fatty acids to some degree into their complex cellular lipids. *A. laidlawii* B is known to extensively utilize a number of exogenous saturated, unsaturated or branched-chain fatty acids for membrane lipid biosynthesis (McElhaney and Tourtellotte, 1969; Romijn *et al.*, 1972). Although the variety of different fatty acids incorporated by this organism is strikingly large, the extent of incorporation can vary greatly depending on the chemical nature and chain length of the exogenous fatty acid. However, the specificity of the enzyme system responsible for the uptake and biosynthetic utilization of exogenous fatty acids has yet to be studied in a detailed and systematic manner. In this chapter I present data on the levels of incorporation of a large variety of exogenous saturated, isobranched, anteisobranched, unsaturated and cyclopropane fatty acids of widely varying chain lengths into the total membrane lipid of *A. laidlawii* B. The object of this study is to determine what physical and chemical properties determine the suitability of an exogenous fatty acid for uptake and incorporation into the membrane lipids of this organism. The possible biological significance of the specificity observed for exogenous fatty acid utilization is discussed. In addition, the fatty acid compositions of each of the major membrane phospho- and glyco-lipids are compared both when A. laidlawii B is grown without fatty acid supplementation and in the presence of various exogenous fatty acids. The metabolic significance of the information obtained from the comparative analysis of the fatty acid compositions of the various membrane polar lipids is also discussed.

A. The effect of the structure of exogenous fatty acids on the extent of incorporation into the total membrane lipids

1. Materials and Methods

Ethanolic solutions containing various fatty acids were added to 125 ml of lipid-poor growth medium before inoculation to give a final fatty acid concentration of 0.12 mM. A. laidlawii B cells were harvested in late-log or early stationary phase after 16 to 20 hours of growth. Lipids were extracted and methyl esters were prepared either by the small scale procedure (when the exogenous fatty acid contained less than 12 carbon atoms) or by the conventional procedure (when the exogenous acid contained 12 or more carbon atoms). The fatty acid composition of the total membrane lipids were determined by GLC. Since this organism continues to synthesize fair amounts of 12:0, 14:0 and 16:0 even in the presence of exogenous fatty acids, the proportion of these three fatty acids which are either incorporated directly from the medium or elongated from exogenous short-chain, even-numbered exogenous saturated fatty acids must be distinguished from those derived by *de novo* biosynthesis. For this purpose exogenous radioactive fatty acids of various chain length were used. Radioactive fatty acids or their sodium salts were mixed with cold carrier acids to bring the final concentration in the growth medium to 0.12 mM. The following are the radioactive compounds: sodium butyrate-1-C^{14} (50 μC), sodium hexanoate-1-C^{14} (50 μC), octanoic acid-1-C^{14} (25 μC), decanoic acid-1-C^{14} (25 μC), dodecanoic acid-1-C^{14} (25 μC), tetradecanoic acid-1-C^{14} (25 μC), and hexadecanoic acid-1-C^{14} (25 μC). Cells were grown and the total membrane lipid extracted as before. The fatty acid composition and the distribution of radioactivity among the membrane lipid fatty acids were simultaneously analyzed with the RAM-GLC system. Small portions, about 4 per cent, of the ethanolic solutions containing the radioactive fatty acid and the unlabeled carrier were directly esterified and analyzed by RAM-GLC under the same conditions used for the analysis of fatty acids in total membrane lipids, to determine the relative response of the RAM-GLC system to the radioactivity and the mass of the exogenous fatty acid precursors. The overall fatty acid compositions in the total membrane lipids, which contain both exogenous and de novo synthesized fatty acids, were determined first from GLC analysis ignoring the RAM pattern. The amount (expressed as mole per cent) of each fatty acid derived from the exogenous fatty acid precursor in the total membrane lipids can be calculated from the following equation:

mole % of a fatty acid derived from an exogenous fatty acid precursor in the total membrane lipids

$$= \text{Area under RAM peak for the acid in question}$$

$$\times \frac{\text{Area under GLC peak for the precursor}}{\text{Area under RAM peak for the precursor}}$$

$$\times \frac{\text{MW of methyl ester of the acid in question}}{\text{MW of methyl ester of the precursor}}$$

$$\times \frac{\begin{array}{l}\text{mole \% of the acid in question} \\ \text{obtained by GLC analysis}\end{array}}{\text{Area under GLC peak for the acid in question}}$$

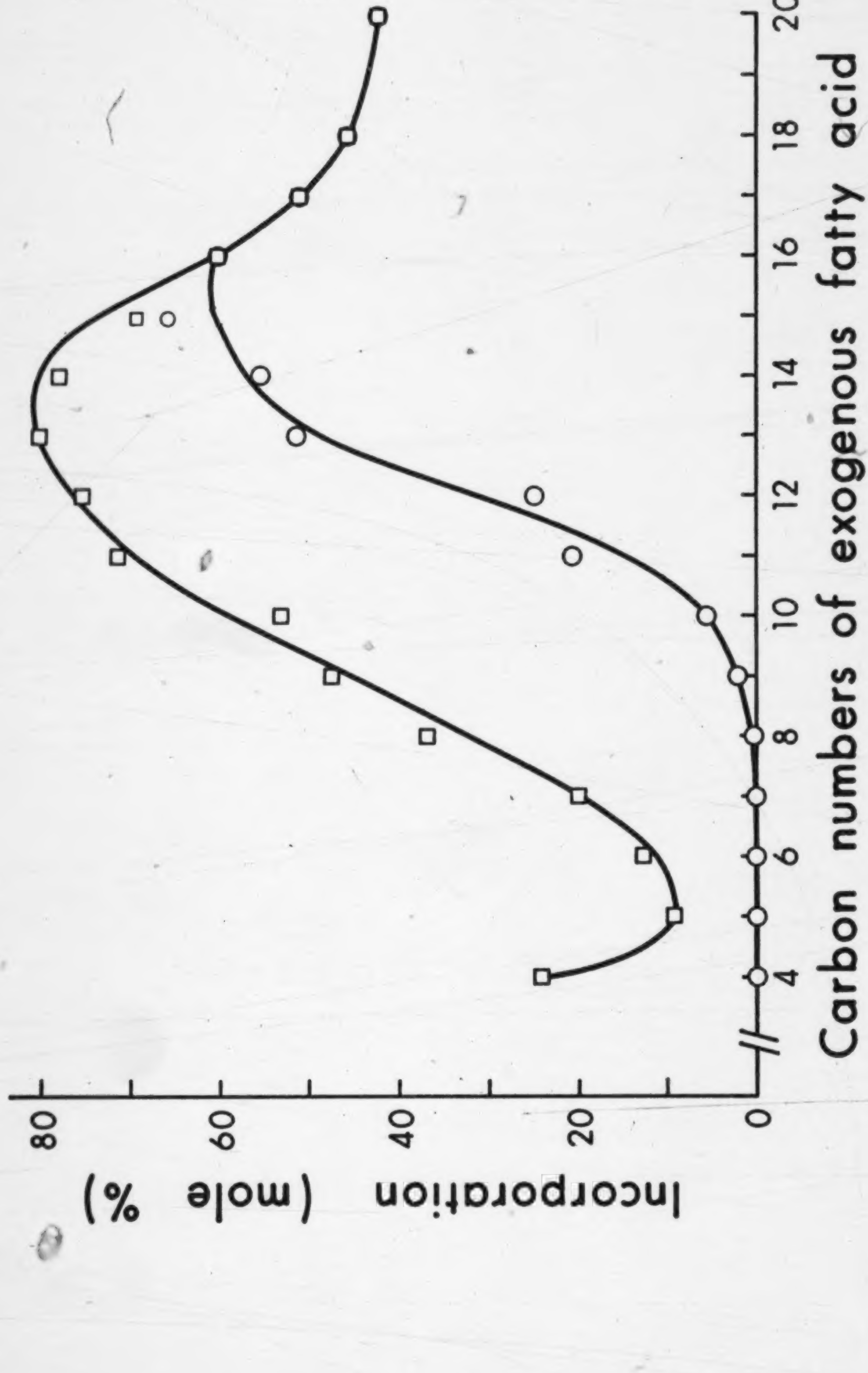
The first two terms of the above equation would give an expected area for the GLC peak of the acid in question, derived from the chain elongation of the precursor, ignoring the difference of the molecular weight between the acid in question and the precursor. The third term of the equation, then, would yield the true expected area for GLC peak of the methyl ester of the acid in question (derived from the chain elongation of the precursor) having the corrected molecular weight. When the ratio of this expected area of the acid in question (derived from the chain elongation of the precursor) to that observed by the GLC analysis (containing both the acid in question de novo synthesized from acetate and elongated from the precursor) are multiplied by the mole per cent of the acid in question (containing both these factors) in the total membrane lipids obtained by the GLC analysis, according to the last term of the equation, the mole per cent of the acid in question derived from chain elongation of the precursos in the total membrane lipids would be obtained. This equation holds even if the precursor is not elongated, which is the case for 16:0.

The concentration of exogenous fatty acids in the growth medium has been fixed at 0.12 mM for the experiments of this section. This concentration was chosen because the amount of fatty acid incorporated into the membrane lipid reaches a maximum value at concentrations of 0.12 mM or less for all exogenous fatty acids studied, except for a few of the shortest-chain fatty acids tested.

2. Results

It was found that some of the exogenous fatty acids were elongated by successive two-carbon unit additions; this chain elongation system is discussed in detail elsewhere in this dissertation. The extent of incorporation of exogenous fatty acids by A. laidlawii B is, therefore, expressed here in two different ways. In the one case, the extent of incorporation is considered to be the amount of a particular exogenous fatty acid which is incorporated into the membrane lipids in a chemically unaltered form (termed direct incorporation). In the second case, the incorporation is defined as the total amount of an exogenous fatty acid and its elongation product which are utilized for complex lipid biosynthesis (termed total incorporation). Fig. 9 shows the effect of chain length on the extent of incorporation of exogenous straight-chain saturated acids of widely varying chain length. Excluding for the moment consideration of possible chain-elongation products, I find that the short-chain acids (4:0 to 8:0) are not directly incorporated into the membrane lipids at all. Increasing the chain length of the exogenous acids, however, progressively increases the amount of the acid directly incorporated until a maximum

Fig. 9. The effect of the chain length of an exogenous straight-chain, saturated fatty acid on the extent of its incorporation into total membrane lipids of *A. laidlawii* B. Cells were grown at 34°C in the presence of one of a series of various straight-chain, saturated fatty acids at a final concentration of 0.12 mM. The total membrane lipids were extracted from late-log or early-stationary phase cells as described in the text. The total and direct incorporation (mole %) of exogenous fatty acids into the total membrane lipids were determined by GLC or RAM-GLC. —□—□— and —○—○— represent the total and direct incorporation values, respectively. The values presented here are the averages of at least triplicate experiments.


Incorporation (mole %)
80
60
40
20
0
4
6
8
10
12
14
16
18
20
Carbon numbers of exogenous fatty acid

incorporation is obtained with 15:0 and 16:0. Increasing the chain length further drastically reduces the extent of incorporation. The data for the total amount of exogenous fatty acid including elongation products exhibit a somewhat different pattern from that observed when only direct incorporation is considered. These differences can be summarized as follows: 1. The curve of the extent of incorporation versus carbon number is broader due to the very extensive elongation of the short-chain acids; 2. the maximum amount of incorporation is obtained with fatty acids containing 13 to 15 carbon atoms, instead of with fatty acids containing 15 or 16 carbon atoms; 3. the maximum amount of short- or intermediate-chain length exogenous fatty acid incorporated is greater due to the chain elongation of these acids to fatty acids containing 13 to 15 carbon atoms; 4. 4:0 is incorporated more extensively than the next three longer chain acids of the saturated series. The differences observed between the total and direct incorporation is most pronounced with shorter chain fatty acids, due to the extensive chain elongation which occurs. In fact, when the chain length of the exogenous acid is less than nine, essentially all of the exogenous acid is converted to longer-chain derivatives. Both iso- and anteiso-methyl branched fatty acids behave very similarly to the straight-chain saturated fatty acids. Data on the extent of incorporation versus chain length for these two fatty acid classes are presented in Fig. 10 and 11 , respectively. Here again differences between the total and direct incorporation are noted due to the elongation of the shorter-chain acids. As before, the chain length giving the maximum total incorporation is shifted downward slightly

Fig. 10. The effect of the chain length of an exogenous isobranched fatty acid on the extent of its incorporation into total membrane lipids of *A. laidlawii* B. Cells were grown at 34°C in the presence of one of a series of various isobranched fatty acids at a final concentration of 0.12 mM. Total membrane lipids were extracted from late-log or early-stationary phase cells as described in the text. The total and direct incorporation (mole %) of the exogenous fatty acids into the total membrane lipids were determined by GLC. -□-□- and -○-○- represent the total and direct incorporation values, respectively. All values presented here are the averages of at least triplicate experiments.

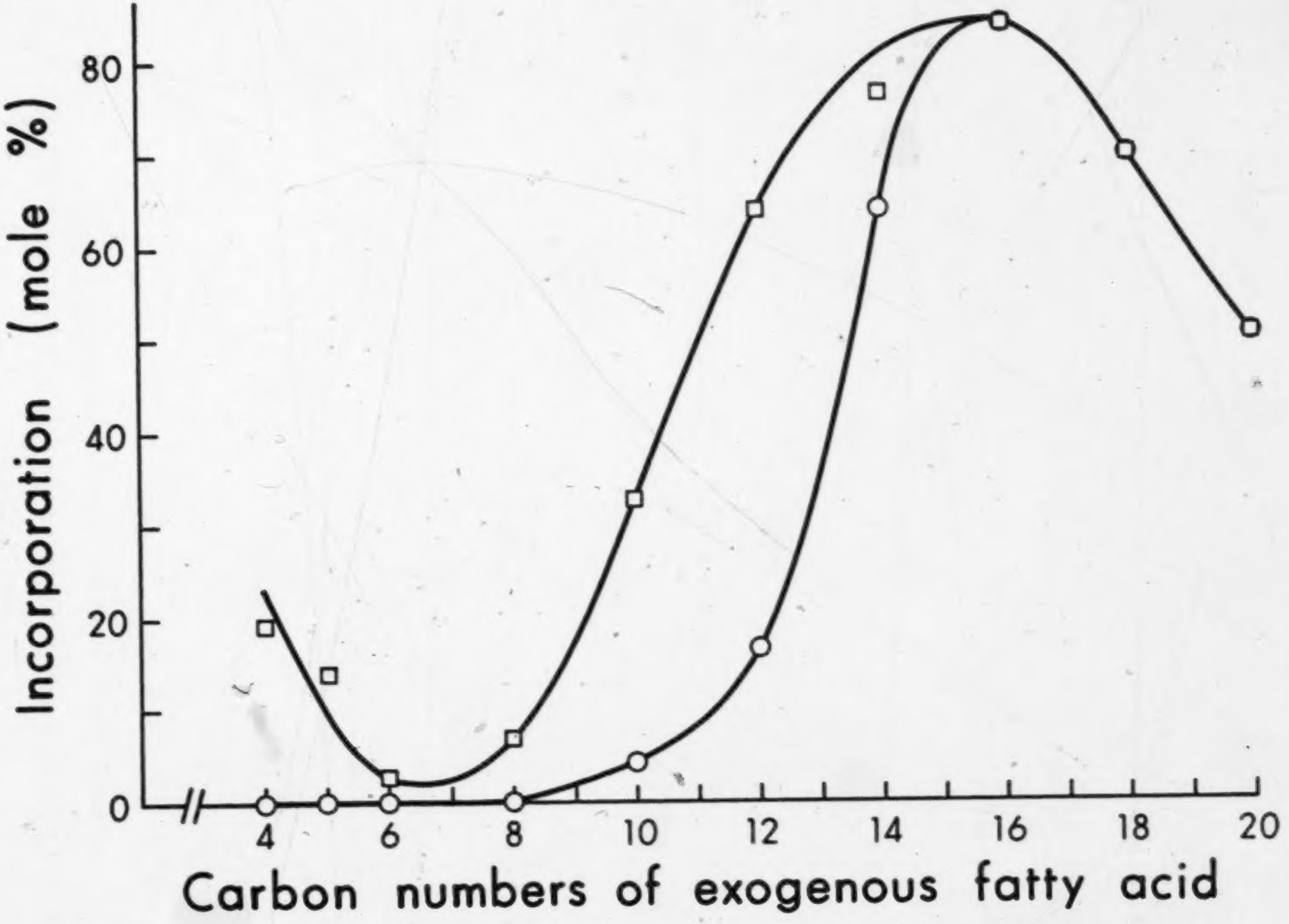
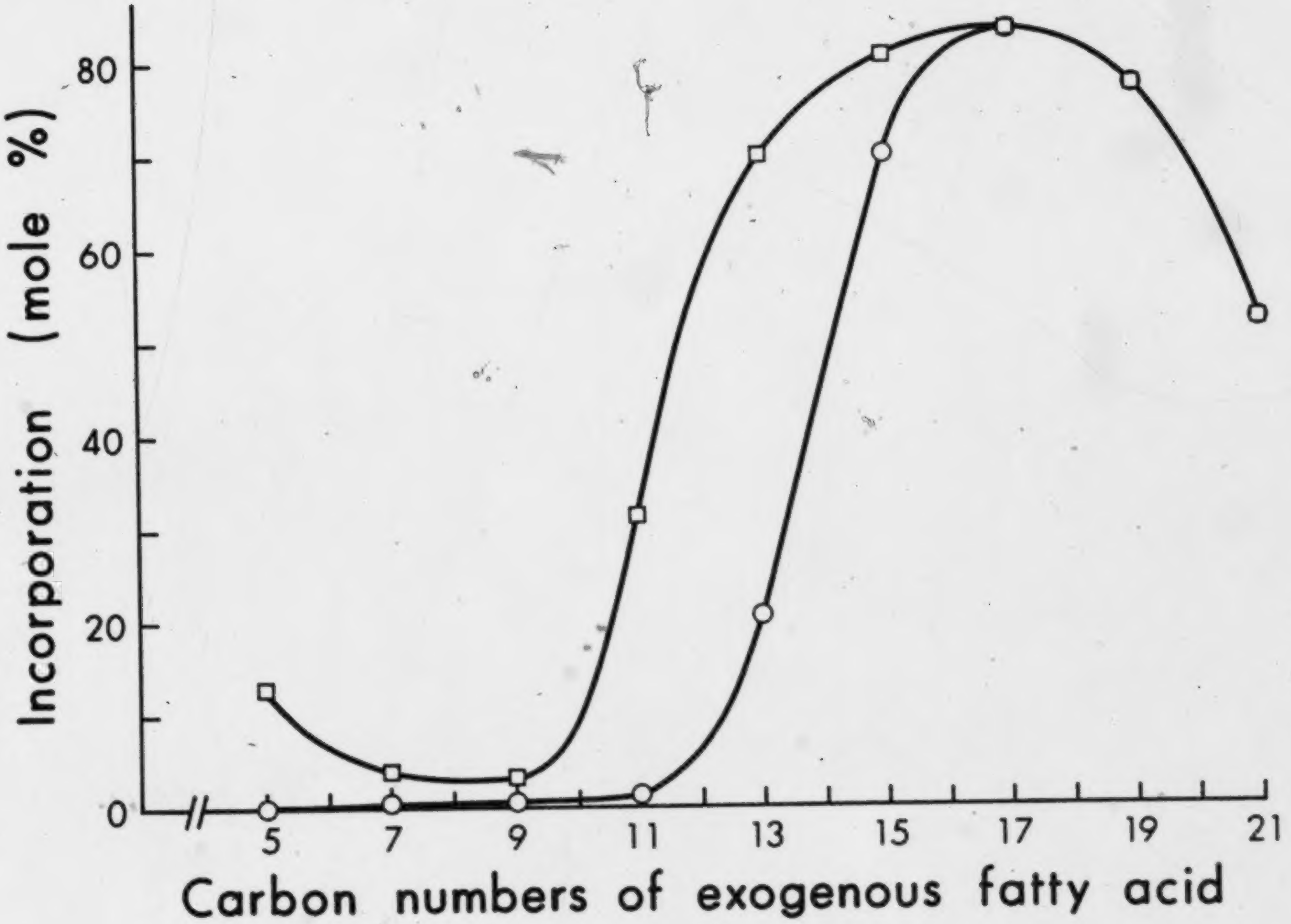

Incorporation (mole %)
0
20
40
60
80
4
6
8
10
12
14
16
18
20
Carbon numbers of exogenous fatty acid

Fig. 11. The effect of the chain length of an exogenous anteiso-branched fatty acid on the extent of its incorporation into total membrane lipids of *A. laidlawii* B. Cells were grown at 34°C in the presence of one of a series of various anteisobranched fatty acids at a final concentration of 0.12 mM. Total membrane lipids were extracted from late-log or early-stationary phase cells as described in the text. The total and direct incorporation (mole %) of the exogenous fatty acids into total membrane lipids were determined by GLC. —□—□— and —○—○— represent the total and direct incorporation values, respectively. All values presented here are the averages of at least triplicate experiments.


Incorporation (mole %)
0
20
40
60
80
5
7
9
11
13
15
17
19
21
Carbon numbers of exogenous fatty acid

relative to the direct incorporation values in the anteisobranched series, although in the isobranched acids 16:0i exhibits both the greatest direct and total incorporation. The introduction of methyl branches into the exogenous fatty acid increases the maximum amount of incorporation relative to the saturated fatty acids and also shifts the chain length giving the maximal incorporation upward. This phenomenon is more clearly demonstrated in Figs. 12 and 13, where data on the effect of the introduction of methyl branching on the direct and total incorporation, respectively, of a series of saturated fatty acids is presented. The chain length for maximum direct incorporation is shifted upward by one and two carbon atoms, for the iso- and anteiso-branched fatty acids, respectively, as compared to the saturated fatty acid series (Fig. 12). The upward shift in the incorporation maxima is even more pronounced when the data for the total incorporation (including elongation products) are inspected for these three fatty acid classes (Fig. 13). We note also that the proportion of exogenous fatty acid which is converted to longer-chain derivatives tends to increase in the order saturated < isobranched < anteisobranched, for a series of fatty acids of comparable chain lengths. Another result of interest concerns the minimum values observed for the total extents of incorporation of the short-chain saturated, isobranched and anteisobranched fatty acids tested. The increased utilization of the very short-chain fatty acids may suggest that there are at least two systems for chain elongation in *A. laidlawii* B; one of them may be responsible for the *de novo* fatty acids (primers) and the other may be for the chain elongation of

Fig. 12. The effect of the chain length of various straight-chain saturated, isobranched and anteisobranched exogenous fatty acids on the extent of direct incorporation into total membrane lipids of *A. laidlawii* B. Cells were grown at 34°C in the presence of one of the various exogenous fatty acids at a final concentration of 0.12 mM. Total membrane lipids were extracted from late-log or early-stationary phase cells as described in the text. The direct incorporation (mole %) of the exogenous fatty acids into the lipids was determined by GLC or RAM-GLC. –□–□– , –○–○– and –△–△– represent straight-chain, isobranched and anteisobranched fatty acids, respectively. All values presented here are the averages of at least triplicate experiments.

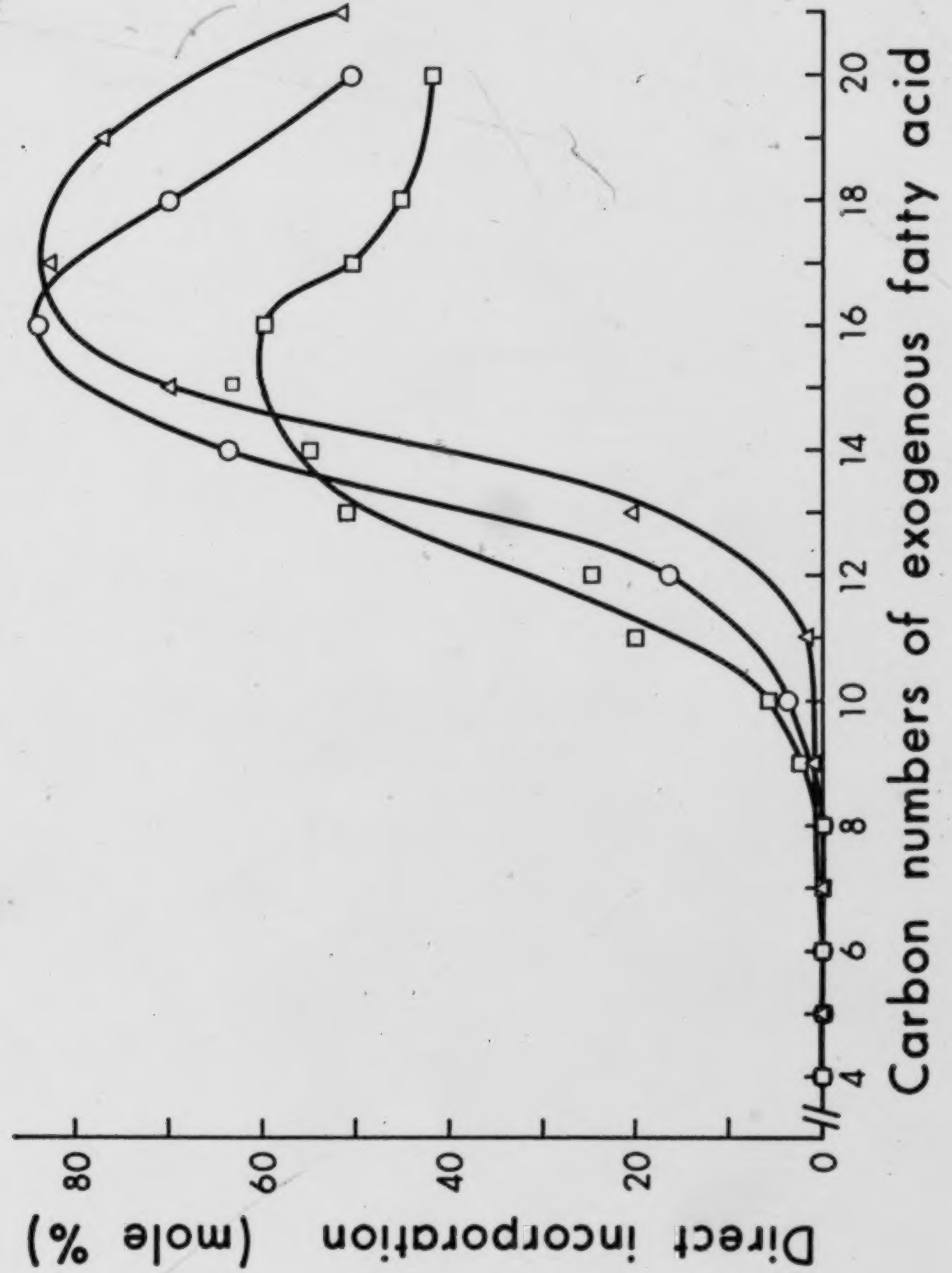
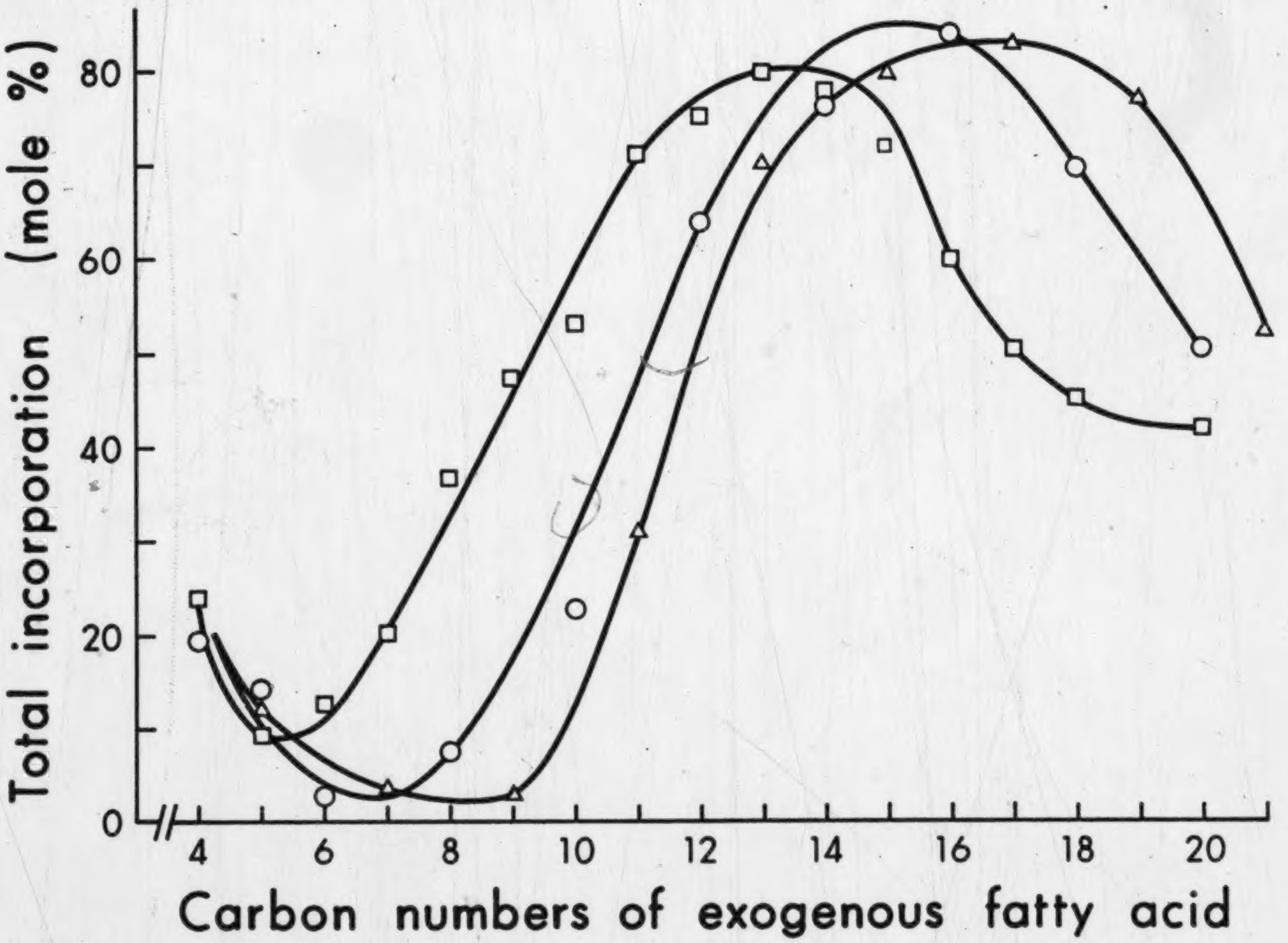

Direct incorporation (mole %)
0
20
40
60
80
Carbon numbers of exogenous fatty acid
4
6
8
10
12
14
16
18
20

Fig. 13. The effect of the chain length of various straight-chain saturated, isobranched and anteisobranched exogenous fatty acids on their total incorporation into total membrane lipids of *A. laidlawii* B. Cells were grown at 34°C in the presence of one of the various exogenous fatty acids at a final concentration of 0.12 mM. Total membrane lipids were extracted from late-log or early-stationary phase cells as described in the text. The total incorporation (mole %) of exogenous fatty acids into the total membrane lipids was determined by GLC or RAM-GLC. —□—□— , —○—○— , and —△—△— represent straight-chain, isobranched, and anteisobranched fatty acids, respectively. All values presented here are the averages of at least triplicate experiments.


Total incorporation (mole %)
0
20
40
60
80
4
6
8
10
12
14
16
18
20
Carbon numbers of exogenous fatty acid

exogenously added acids of intermediate chain length. This is further discussed elsewhere in this dissertation in conjunction with a study of the chain elongation of exogenous fatty acids by this organism.

The effect of the introduction of unsaturation or a cyclopropane ring on the extent of incorporation of an exogenous fatty acid is presented in Table 11. Quite a big difference between the direct and total incorporation is again obvious, especially for the short chain cis-monounsaturated acids, 9-14:1c and 9-16:1c, again due to the extensive elongation of these acids. When the cis-monounsaturated series of fatty acids are examined, the extents of total incorporation are constant (about 70 mole per cent) from 9-14:1c to 9-18:1c. Increasing the chain length beyond 18 carbon atoms abruptly reduces the incorporation. The longest cis-monounsaturated fatty acid tested, 15-24:1c, is incorporated to only 16 mole per cent. The amount of 9-14:1c directly incorporated is roughly half that observed with the corresponding saturated acid, 14:0, but the total incorporation is only slightly less due to the extensive elongation of the former. The 9-16:1c is directly incorporated to a level only about 60 per cent of that of the corresponding saturated acid, 16:0, but the total incorporation of the former slightly exceeds the latter because of the high degree of chain elongation of this unsaturate. The longer chain cis-monounsaturated fatty acids, 9-18:1c and 11-20:1c, on the other hand, are directly incorporated to a greater extent than the corresponding saturated acids. It should be noted that the chain length of the cis-monounsaturate which gives maximum direct incorporation is in the range of 18 to 19 carbon atoms. The presence of a single

Table 11.[1] The extent of direct and total incorporation of exogenous unsaturated and cyclopropane ring-containing fatty acids into total membrane lipids of *A. laidlawii* B

Exogenous fatty acid	Direct incorporation (mole %)	Total incorporation (mole %)
cis-monounsaturated		
9-14:1c	28.6[a]	72.6[a]
9-16:1c	36.2	69.9
9-18:1c	68.9	71.7
11-20:1c	58.3	58.3
13-22:1c	36.6	36.6
15-24:1c	15.9	15.9
trans-monounsaturated		
9-14:1t	64.3	83.9
9-16:1t	78.8	79.7
9-18:1t	82.4	82.4
cis-polyunsaturated		
9,12-18:2c,c	47.6	57.3
9,12,15-18:3c,c,c	36.4	56.3
5,8,11,14-20:4c,c,c,c	31.7	31.7
Cyclopropane		
9,10-17:0cp,c	58.3	66.7
9,10-19:0cp,c	57.8	57.8
9,10-19:0cp,t	67.4	67.4
Straight-chain		
14:0	55.4	77.9
16:0	60.4	60.4
18:0	45.2	45.2
20:0	43.0	43.0

[a] These values are the average of at least triplicate experiments.

cis-double bond thus shifts the incorporation optimum to an even greater chain length as compared to the corresponding isobranched and anteisobranched fatty acid series.

All the trans-monounsaturated fatty acids investigated are incorporated to a greater extent than the corresponding cis-monounsaturated acids, the extent of total incorporation being constant at about 80 mole per cent. The direct incorporation of 9-14:1t and 9-16:1t is more than twice that of the corresponding cis-monounsaturated acids, due to both the better incorporation and the smaller amount of elongation of the trans-unsaturates. The cis-polyunsaturated fatty acids tested are relatively poorly incorporated compared to the other fatty acid classes tested. The introduction of increasing numbers of cis-double bonds considerably reduces the extent of incorporation. Cyclopropane ring-containing fatty acids are fairly well utilized, and the amounts incorporated are very close to that of corresponding cis-monounsaturated acids, but considerably lower than that of the corresponding trans-monounsaturates. Comparing cyclopropane ring-containing acids having the same chain length, the trans isomer is again incorporated to a greater extent than is the cis isomer.

The effect of the introduction of various functional groups into the exogenous fatty acids on the extent of incorporation is summarized in Table 12. When the chain length is 14, the introduction of isobranching, anteisobranching and trans-monounsaturation increases moderately both the direct and total incorporation. However, the introduction of cis-monounsaturation greatly reduces the direct incorporation, and slightly reduces the total incorporation. When the

Table 12. The effect of the introduction of various functional groups into an exogenous fatty acid on the extent of its incorporation (mole %) into the total membrane lipid of A. laidlawii B

	Number of carbon atoms in the fatty acid molecule			
Exogenous fatty acid	14 (mole %)	16 (mole %)	18 (mole %)	20 (mole %)
Straight-chain	55.4 (77.9)[a]	60.4	45.2	43.0
isobranched	63.8 (76.6)	87.6	69.8	54.1
anteisobranched[b]	74.1 (80.8)	84.0	77.4	52.0
cis-monounsaturated	28.6 (72.6)	36.2 (69.9)	68.9(71.7)	58.3
trans-monounsaturated	64.3 (83.9)	78.8 (79.7)	82.4	-
cis-polyunsaturated	-	-	47.6 (57.3)[c] 36.3 (56.3)[e]	31.7[d]
cyclopropane	-	58.3 (66.7)[f]	57.8[g] 67.4[h]	-

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[a] The figures before the parentheses represent the direct incorporation and the figures within the parentheses the total incorporation, if any chain elongation of the exogenous fatty acid occurred. Otherwise, one figure represents both the direct and total incorporation values. The values presented here are the averages of at least triplicate experiments.

[b] The total carbon number of all exogenous anteisobranched fatty acids is greater by one than is indicated in the table.

[c] 9,12-18:2c,c

[d] 5,8,11,14-20:4c,c,c,c

[e] 9,12,15-18:3c,c,c

[f] 9,10-17:0cp,c

[g] 9,10-19:0cp,c

[h] 9,10-19:0cp,t

chain length is 16 the introduction of isobranching, anteisobranching, cis-monounsaturation, trans-monounsaturation and a cyclopropane ring enhances the total incorporation, whereas both the introduction of a cis-double bond or cyclopropane ring reduces the direct incorporation. When the chain length is 18 the introduction of all of these functional groups enhances both the total and direct incorporation. If the chain length is 20 the introduction of isobranching, anteisobranching, and cis-monounsaturation increases the incorporation. The introduction of four cis-double bonds, however, gives somewhat reduced incorporation.

3. Discussion

The cohesive force among fatty acyl chains in the membrane lipid bilayer is dependent upon the length and the nature of the hydrocarbon chain. Increasing the number of methylene groups increases the cohesive force. Since van der Waals attractive forces are inversely proportional to the sixth power of the distance between the two interacting atoms, the introduction of chemical groups, which inhibit the close-packing characteristic of straight-chain saturated fatty acids, significantly lowers the cohesive force. The introduction of isobranching, trans-monounsaturation, anteisobranching, cis-monounsaturation, a cis-cyclopropane-ring, and cis-polyunsaturation into a straight saturated acid would disrupt the close packing and progressively lower the cohesive force, roughly in that order. The reduced cohesive force will greatly enhance the fluidity of the hydrocarbon core of the membrane.

The data for the direct incorporation of exogenous fatty acids reveals that these acids must have the proper range of chain lengths in order to be effectively utilized for membrane lipid synthesis. When grown in the absence of exogenous acids, this organism synthesizes roughly equal amounts of 14:0 and 16:0, as discussed in the first chapter of this dissertation. This fatty acid composition presumably gives a "natural" and proper fluidity to the hydrocarbon core of the membrane. When a whole series of straight-chain saturated acids are examined, this organism directly incorporates 14:0, 15:0 and 16:0 more extensively into membrane lipids compared to fatty acids having longer- or shorter-chain lengths. The introduction of methyl branching should reduce the "apparent chain length", because the introduction of methyl branching into the hydrocarbon portion of a fatty acid reduces somewhat the nonpolar attractive forces developed between the interacting fatty acids. The anteisobranching should have a larger effect than the corresponding isobranching in reducing the cohesive forces between fatty acyl chains. Therefore, one would predict that the chain lengths giving maximum incorporation would be greater for the isobranched and greater still for the anteisobranched fatty acids, which is exactly what is observed in Fig. 13. Although a comparable series of cis- and trans-monounsaturated fatty acids were not available for testing, a similar upward shift in the optimal chain length for incorporation would be predicted for the introduction of double bonds. Again on the basis of the magnitude of nonpolar cohesive interactions, one would expect that the cis-double bond would shift the optimal chain length to the greatest extent, while the trans-double bond would produce a shift roughly comparable to the introduction of an anteiso-methyl branch. Although the optimal chain length of the trans-monounsaturates can not be determined precisely, the predictions seem to be borne out by the data (Table 11). If the chain length is shorter than 12 to 14 carbon atoms, the introduction of any one of these functional groups will reduce the incorporation, because they will reduce the apparent chain length, which is already shorter than the proper range. Whereas if the chain length is more than 14 to 16 the introduction of those groups will enhance the incorporation, because they will reduce the apparent chain length which otherwise would be longer than the proper range. However, the introduction of two or more cis-double bonds apparently reduces the apparent chain length of 18 and 20 carbon fatty acids by too great an extent, since the uptake of polyunsaturates markedly declines with increasing unsaturation. Apparently an upper as well as a lower limit for the fluidity of the membrane lipids exists in this organism. It is interesting to note that, when the chain length is optimized, the iso- and anteiso-branched and the trans-monounsaturated fatty acid classes are incorporated more extensively than are members of the other fatty acid classes. It is tempting to speculate that this behavior is due to the fact that these classes of fatty acids can provide a moderate and optimal level of membrane lipid fluidity, being intermediate in this regard between the high-melting saturated and lower-melting cis-monounsaturated, cyclopropane, and polyunsaturated fatty acids. In conjunction with this idea, it might be noted that 9-18:1t was the only exogenous fatty acid capable of supporting cell growth of Mycoplasma strain Y, when this organism, which has an absolute requirement for exogenous fatty acids, was maintained on a series of single saturated, or unsaturated fatty acids (Rodwell, 1969). Unfortunately, branched-chain fatty acids were not tested in these experiments.

The chain length specificity for the total incorporation of exogenous fatty acids by A. laidlawii B is wider than that for direct incorporation due to the chain elongation. If the chain length of exogenous fatty acids is shorter than 10, 12 and 13 carbon atoms, when they are straight-chain saturated, isobranched, and anteisobranched acids, respectively, virtually all the exogenous acids are elongated to fatty acids longer than 10:0, 12:0i, and 13:0ai before being utilized for complex lipid biosynthesis. From the data on the total incorporation, exogenous fatty acids can be classified into four different groups. The first group of fatty acids seems to have the proper range of physicochemical properties without chemical modification. Fatty acids in this group are directly incorporated most extensively into the membrane lipids and are elongated by two carbon additions little if at all. This group includes straight-chain saturated acids containing 13 to 15 carbon atoms, isobranched acids of 14 to 18 carbon atoms, anteisobranched fatty acids of 15 to 19 carbon atoms, and the 14 to 18 carbon trans-monounsaturated acids. The second group of acids has a potentially large disruptive effect on the packing and order of the hydrocarbon chains but can be elongated to reduce this effect by shifting the average chain length toward the optimum. The short-chain saturated, branched-chain and monounsaturated fatty acids belonging to this second group are incorporated moderately to well. The third group of exogenous fatty acids are also characterized by high degrees of fluidity but are apparently not suitable substrates for chain elongation. Members of this third group of fatty acids, which includes the cyclopropane and polyunsaturated fatty acids, are relatively poorly incorporated. The last group of acids are ones which are characterized by too high or too low a degree of cohesive interaction. The long-chain saturated fatty acids and a polyunsaturated acid (5,8,11,14-20:4c,c,c,c) which make up this fourth group are the least extensively incorporated of all the exogenous fatty acids tested.

The chemical nature of the exogenous fatty acid does not seem to be of major importance in determining its suitability for biosynthetic utilization, since no correlation between the degree of incorporation and the presence of any particular functional group or electronic configuration could be discerned. Also, the marked dependence of incorporation on chain length within any chemical class of exogenous fatty acids supports this view. Instead, the physical properties of the exogenous fatty acids, and in particular their melting points, appear to be the basis of incorporation selectivity. The evolutionary development of an enzyme system capable of utilizing a wide variety of chemically dissimilar but physicochemically related fatty acids for membrane lipid biosynthesis probably provided a significant adaptational advantage to A. laidlawii B. In the presence of group one fatty acids, the bulk of the membrane lipid fatty acids can be directly derived from exogenous sources, so that this organism can maintain its rate of membrane growth with only a fraction of the metabolic energy that would be required in the absence of exogenous fatty acids. The extensive utilization of exogenous fatty acids belonging to groups one and two does not excessively alter the physical state of the membrane lipids. The incorporation of exogenous fatty acids belonging to groups three and four is much less extensive, probably because extensive utilization of those rather low-melting and high-melting fatty acids would perturb the physical state of the membrane lipids to too great a degree. Thus the enzyme system which incorporates exogenous fatty acids in this organism has a broad but definite specificity, and seems to function to maintain an optimal range of membrane lipid fluidity by controlling the physicochemical properties of the membrane lipid fatty acyl groups. The degree of incorporation of exogenous fatty acids which do not possess the optimal physicochemical properties is doubtlessly increased by the compensatory shifts in the chain length specificity of the *de novo* fatty acid biosynthetic system, as discussed earlier. The conclusion that it is the physical, and not the chemical, properties of an exogenous fatty acid which are important in determining its suitability for biosynthetic utilization strongly argues against the membrane model proposed by Benson (1966). This model requires that the hydrocarbon chain of the membrane lipids be specifically complementary to certain hydrophobic amino acid sequences in the membrane protein and that each class of lipids have a specific and characteristic fatty acid spectrum. If this model system were correct for the membrane of *A. laidlawii* B, the membrane lipids should not be able to incorporate such a wide range of chemically different fatty acyl groups. The discovery of unsaturated fatty acid auxotrophs of *E. coli* (Silbert and Vagelos, 1967) suggested that this organism had an essential requirement for certain types of fatty acyl groups in its membrane lipid. However, a variety of fatty acids have been shown to be able to replace _cis_-monounsaturated fatty acids as essential growth factors for these unsaturated fatty acid auxotrophs. These are _cis_-polyunsaturated, _trans_-monounsaturated, 12-bromostearic, and cyclopropane-ring containing acids (Silbert _et al._, 1968; Esfahani _et al._, 1969, 1971b; Schairer and Overath, 1969; Fox _et al._, 1970). Further studies by Silbert _et al._ (1973a) have demonstrated that even methyl-branched acids, both iso- and anteiso-branched fatty acids, which have dissimilar electronic configuration, can replace _cis_-monounsaturated fatty acids as the growth factors in a derivative of this mutant. They have concluded that unsaturated fatty acids do not participate in a specific interaction with other membrane components but serve primarily to control the packing of paraffin chains and the fluidity of the membrane bilayer (Silbert _et al._, 1973a, 1974).

The incorporation of exogenous fatty acids into membrane lipids is a result of a very complex series of biological reactions including the interaction of free fatty acids with BSA in the medium, the transport of fatty acids across the cell membrane into the cell, the activation of free acids to CoA- or ACP-derivatives, possibly chain elongation which itself includes several steps, and the incorporation of the activated fatty acid to the membrane polar lipids. From this overall _in vivo_ experiment alone it is very difficult to tell which steps are responsible for the specificity pattern observed. Further studies should be conducted to clarify this point. In connection with the question of where the observed specificity is localized, Klein et al. (1971) have postulated that fatty acid transport in E. coli might be mediated by the fatty acid activating enzyme, acyl-CoA synthetase, and have introduced the term "vectorial acylation" to describe the fatty acid transport system in this organism. They have shown that E. coli actively transports intermediate- and long-chain acids including 9-18:1c, but that the transport of 4:0 and 6:0 are almost negligible. They have also shown that the transport process is irreversible and very tightly linked to the fatty acid metabolism. The fatty acid transport by Pseudomonas oleovorans (Toscano and Hartline, 1973) has been shown to be saturable and chain length-specific, with short-chain fatty acids being excluded. Brindley (1973) has shown that in the microsomal fraction from guinea-pig intestinal mucosa there is a fine balance between the activities of 16:0-CoA synthetase and glycerol phosphate acyltransferase.

It is possible that the exogenous fatty acids could be transported into A. laidlawii B cells via vectorial acylation by acyl-CoA synthetase which is closely linked to either the direct acylation and/or the chain elongation system, if necessary. These three closely linked activities might control the fluidity of the membrane lipids of this organism. On the other hand, since the passive flux of the hydrophobic intermediate and long-chain exogenous fatty acids across the cell membrane should be appreciable, one need not postulate a fatty acid transport system in this rather slow-growing organism, which also can not oxidize exogenous fatty acids. The specificity for the incorporation of exogenous fatty acids by A. laidlawii B probably resides in the activation and esterification reactions of the complex lipid biosynthetic pathway.

B. Incorporation of exogenous fatty acids into various membrane complex lipids

1. Materials and Methods

Ethanolic solutions of various fatty acids, which would be elongated little if at all, were added to 1 l quantities of lipid-poor growth medium before inoculation to a final concentration of 0.03 to 0.12 mM, such that the exogenous fatty acid would comprise about 50 mole per cent of the fatty acids in the membrane lipids. The cells were harvested in late-log or early-stationary phase, and total lipids were extracted and separated into individual complex lipids by a combination of column chromatography and TLC. The total lipids were first separated into seven fractions by Unisil column chromatography by a modification of the method of Vorbeck and Marinetti (1965). Unisil (10 g) was packed into a column in about 50 ml of hexane, and washed successively with 75 ml of hexane, 60 ml of diethylether, and 150 ml of chloroform in that order. Fifty ml each of the following solvents were successively used to elute neutral lipids and fractions I to VII, respectively: chloroform (which would elute neutral lipids), chloroform-acetone (2:3, vol./vol.), acetone, chloroform-methanol (4:1), chloroform-methanol (3:1), chloroform-methanol (7:3), chloroform-methanol (6.5:3.5), and methanol. The neutral lipids fraction was discarded. Fractions I to VII were further purified by TLC. TLC plates, 5 x 20 cm, 0.5 mm thick, were prepared from silica gel H. The solvent used to purify fractions I and II was chloroform-methanol-water (75:25:3) and the solvent used for fractions III to VII was chloroform-methanol-water (65:25:4). The position of the various lipids on the TLC plates was visualized by briefly exposing the plates to iodine vapor. After removal of the iodine stain by sublimation in a vacuum desiccator, areas of gel containing each fraction were scraped from the TLC plates, packed into a small column, and eluted with 10 ml methanol. Five different major lipids were obtained from this purification procedure. Fatty acid methyl esters were prepared from each lipid class by the conventional procedure and the fatty acid compositions were analyzed by GLC.

2. Results

The purity of the five extracted lipids was checked by TLC, and the results are shown in Fig. 14. The solvent system used was chloroform-methanol-water (65:25:4). The Rf values obtained are very close to those previously reported by Shaw et al. (1968). The five classes of lipids are identified as follows, based on my own analytical data and that of Shaw et al. (1968, 1972):

A - Monoglucosyl diglyceride (MGDG)
B - Diglucosyl diglyceride (DGDG)
C - Phosphatidylglycerol (PG)
D - Glycerophosphoryldiglucosyl diglyceride (GPDGDG)
E - O-Amino acyl ester of phosphatidylglycerol (O-PG)

The chemical structures of these lipids are shown in Fig. 15.

When cells are grown in the absence of any exogenous fatty acid, the membrane lipid fatty acids, which are derived mainly from

Fig. 14. A thin-layer chromatogram illustrating the separation of the five major membrane polar lipids of A. laidlawii B on Silica Gel H. The solvent system used for the development of the TLC chromatoplate was chloroform-methanol-water (65:25:4 by vol.). A, B, C, D and E are the monoglucosyl diglyceride (MGDG), diglucosyl diglyceride (DGDG), phosphatidylglycerol (PG), glycerophosphoryl-diglucosyl diglyceride (GPDGDG) and O-amino acyl ester of phosphatidyl glycerol (O-PG), respectively. PG is a commercial phosphatidylglycerol standard.

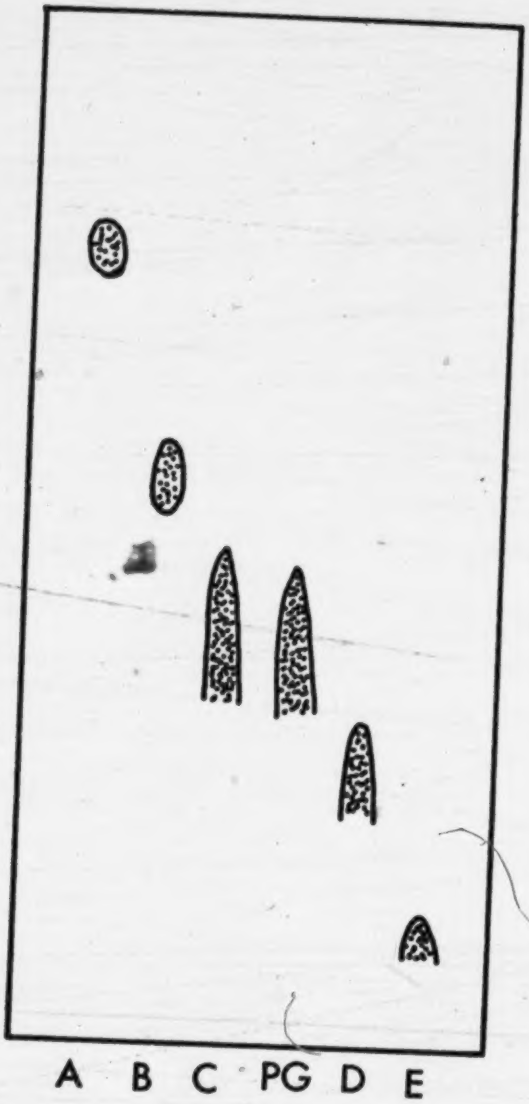
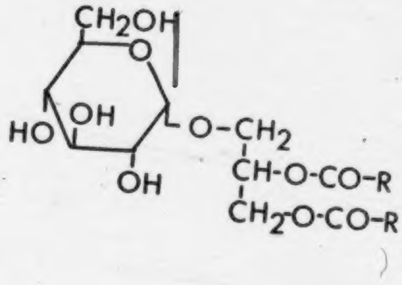
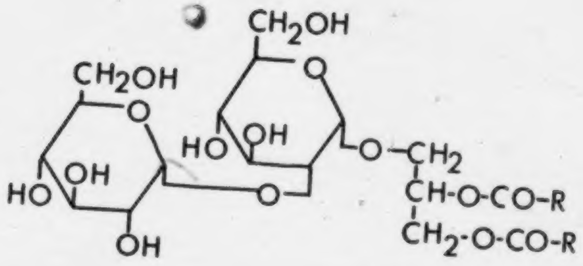

A
B
C
PG
D
E

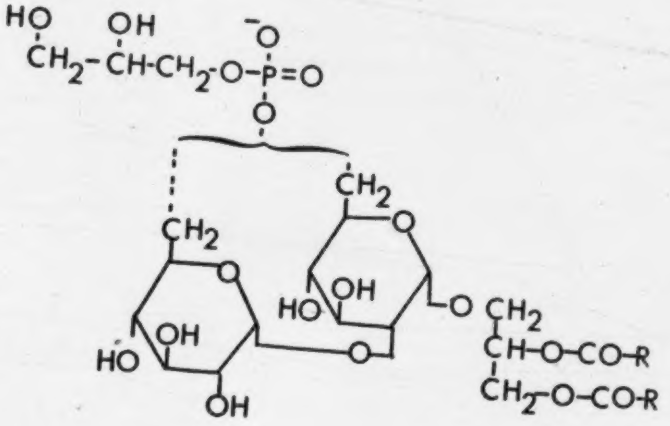
Fig. 15. The chemical structures of the five major membrane polar lipids of A. laidlawii B. A, B, C, D and E are monoglucosyl diglyceride, diglucosyl diglyceride, phosphatidylglycerol, glycerophosphoryldiglucosyl diglyceride, and O-amino acyl ester of phosphatidylglycerol, respectively.

A

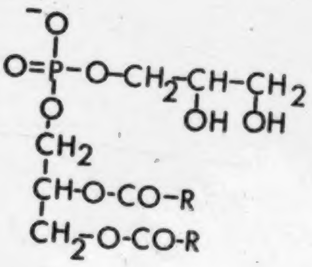


B

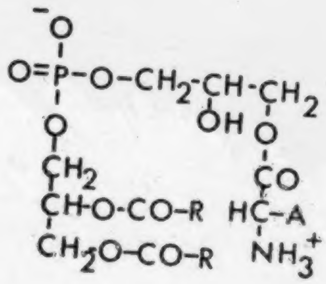




D



C



E

de novo biosynthesis, are distributed among the various lipid classes in a rather distinctive manner, as shown in Fig. 16. The fatty acid compositions of the two neutral glycolipids, MGDG and DGDG, are very similar and somewhat different from that of the phospholipids. The fatty acid composition of the two phospholipids, PG and O-PG, are also very similar. However, the phosphorylated glycolipid, GPDGDG, has a rather different and distinctive fatty acid composition, although it more closely resembles the neutral glycolipids. GPDGDG has a much higher content of 12:0, and less 16:0 and 18:0 than any of the other fractions. In order to determine whether or not these differences in fatty acid composition would persist when the bulk of the membrane lipid fatty acids were derived from exogenous sources, the fatty acid composition of each lipid class from cells grown in a variety of exogenous fatty acid was studied. The pattern of fatty acid composition noted with the endogenous fatty acids holds with certain exogenous fatty acids, but it does not with others. However, any systematic pattern of distribution of exogenous fatty acids among the various lipid classes has not been discernible in the present experiments. In some cases, represented here in Fig. 17 by an experiment using 17:0 as the exogenous acid, the distinctive differences between the neutral glycolipids and phospholipids are retained. GPDGDG again shows the very peculiar pattern noted previously. In other cases, represented here in Fig. 18 by an experiment using 17:0ai as the exogenous acid, those distinctive differences between the neutral glycolipids and phospholipids are no longer observed. GPDGDG, however, still shows the peculiar pattern.

Fig. 16. The distribution of the major fatty acids within the five major membrane polar lipids of A. laidlawii B grown in the absence of exogenous fatty acids. The cells were grown at 34°C in the absence of exogenous fatty acids. The five major lipid classes were separated, by the method described in the text, from the total membrane lipids extracted from the late-log or early-stationary phase cells. The fatty acid composition (mole %) of each lipid class was determined by GLC. A, B, C, D and E are MGDG, DGDG, PG, GPDGDG, and O-PG, respectively. The values presented here are the averages of triplicate experiments.

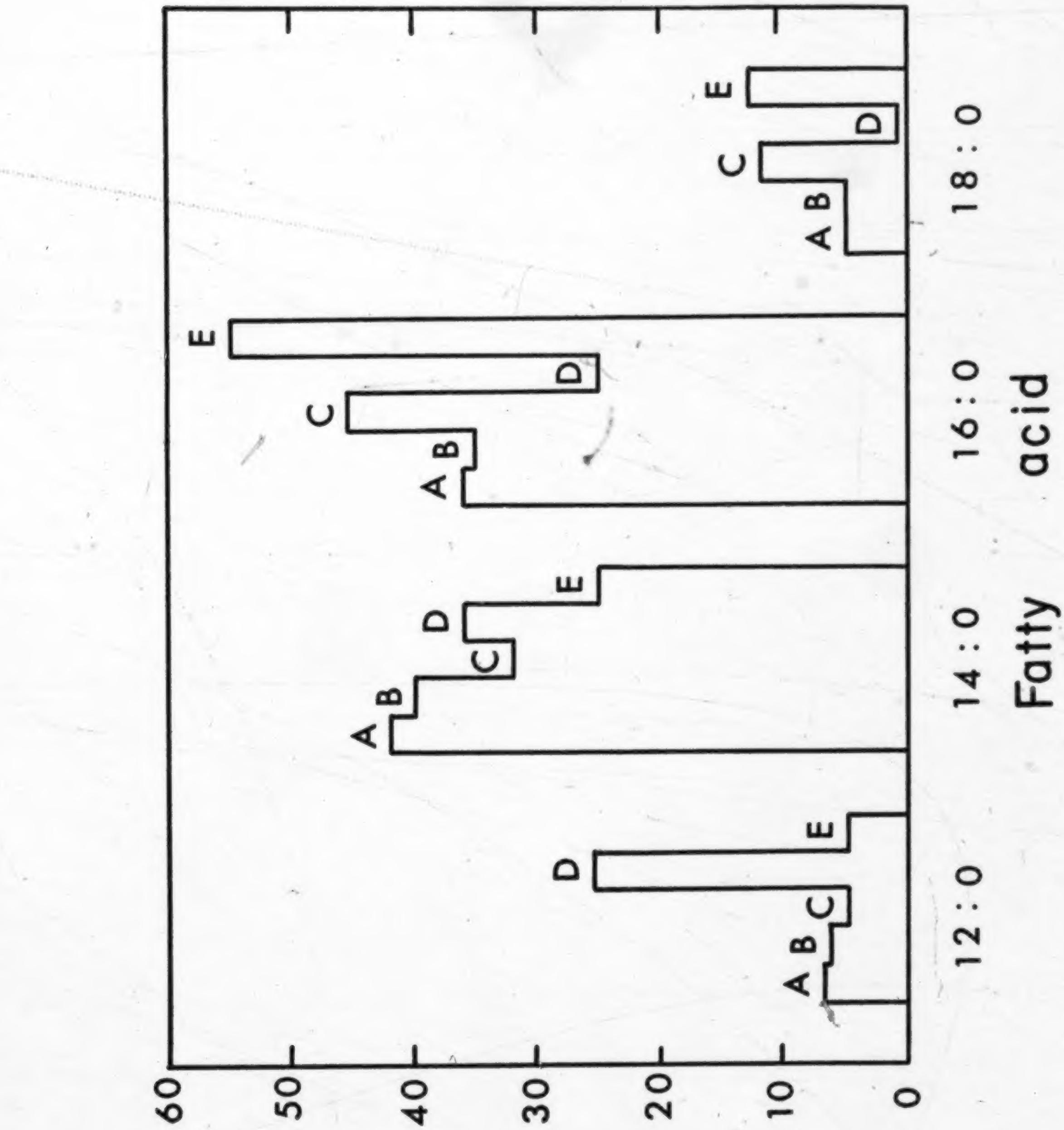

mole %
0
10
20
30
40
50
60
12 : 0
14 : 0
16 : 0
18 : 0
Fatty acid
A
B
C
D
E

Fig. 17. The distribution of the major fatty acids within the five major membrane polar lipids of A. laidlawii B grown in the presence of 17:0. The cells were grown at 34°C in the presence of an exogenous fatty acid, 17:0, at a final concentration of 0.10 mM. The five major lipid classes were separated by the method described in the text from the total membrane lipids extracted from late-log or early-stationary phase cells. The fatty acid composition (mole %) of each lipid class was determined by GLC. A, B, C, D and E are MGDG, DGDG, PG, GPDGDG and O-PG, respectively. The values presented here are representative of duplicate experiments.

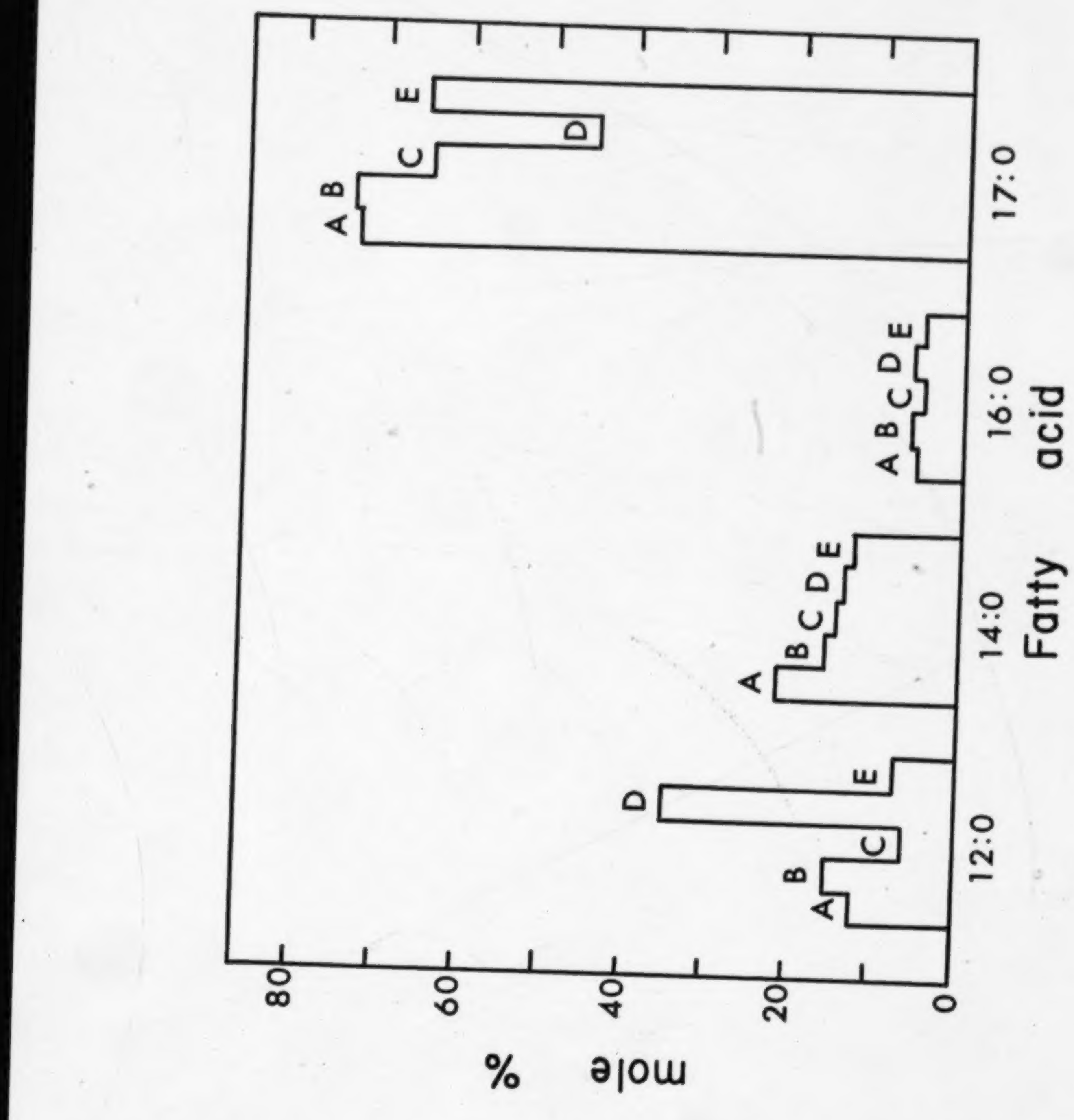
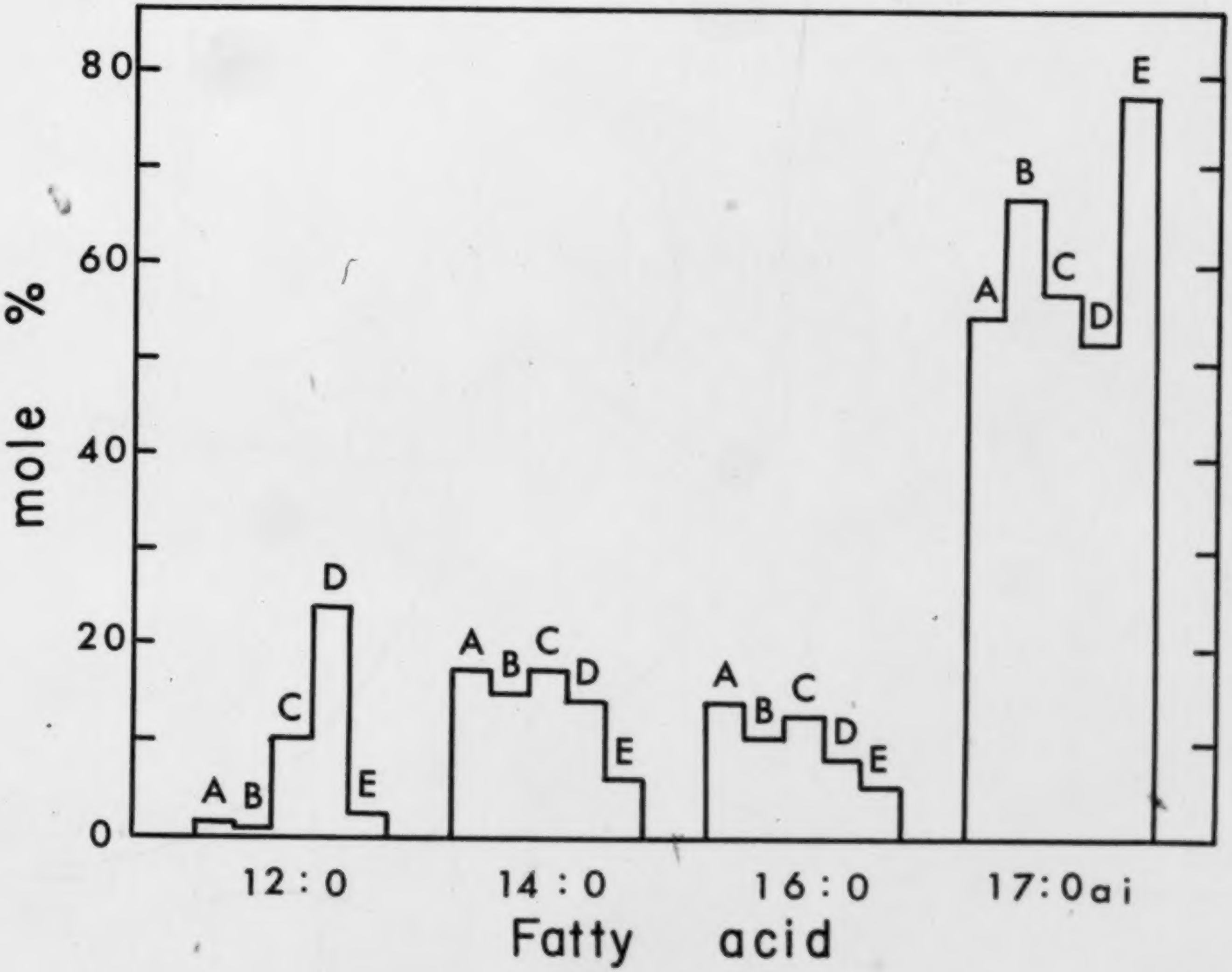

mole %
0
20
40
60
80
Fatty acid
12:0
14:0
16:0
17:0
A
B
C
D
E

Fig. 18. The distribution of the major fatty acids within the five major membrane polar lipids of A. laidlawii B grown in the presence of 17:0ai. The cells were grown at 34°C in the presence of an exogenous fatty acid, 17:0ai, at a final concentration of 0.03 mM. The five major lipid classes were separated by the method described in the text from the total membrane lipids, extracted from late-log or early-stationary phase cells. The fatty acid composition (mole %) of each lipid class was determined by GLC. A, B, C, D and E are MGDG, DGDG, PG, GPDGDG and O-PG, respectively. The values presented here are representative of duplicate experiments.



mole %
80
60
40
20
0
A
B
C
D
E
12:0
14:0
16:0
17:0ai
Fatty acid

3. Discussion

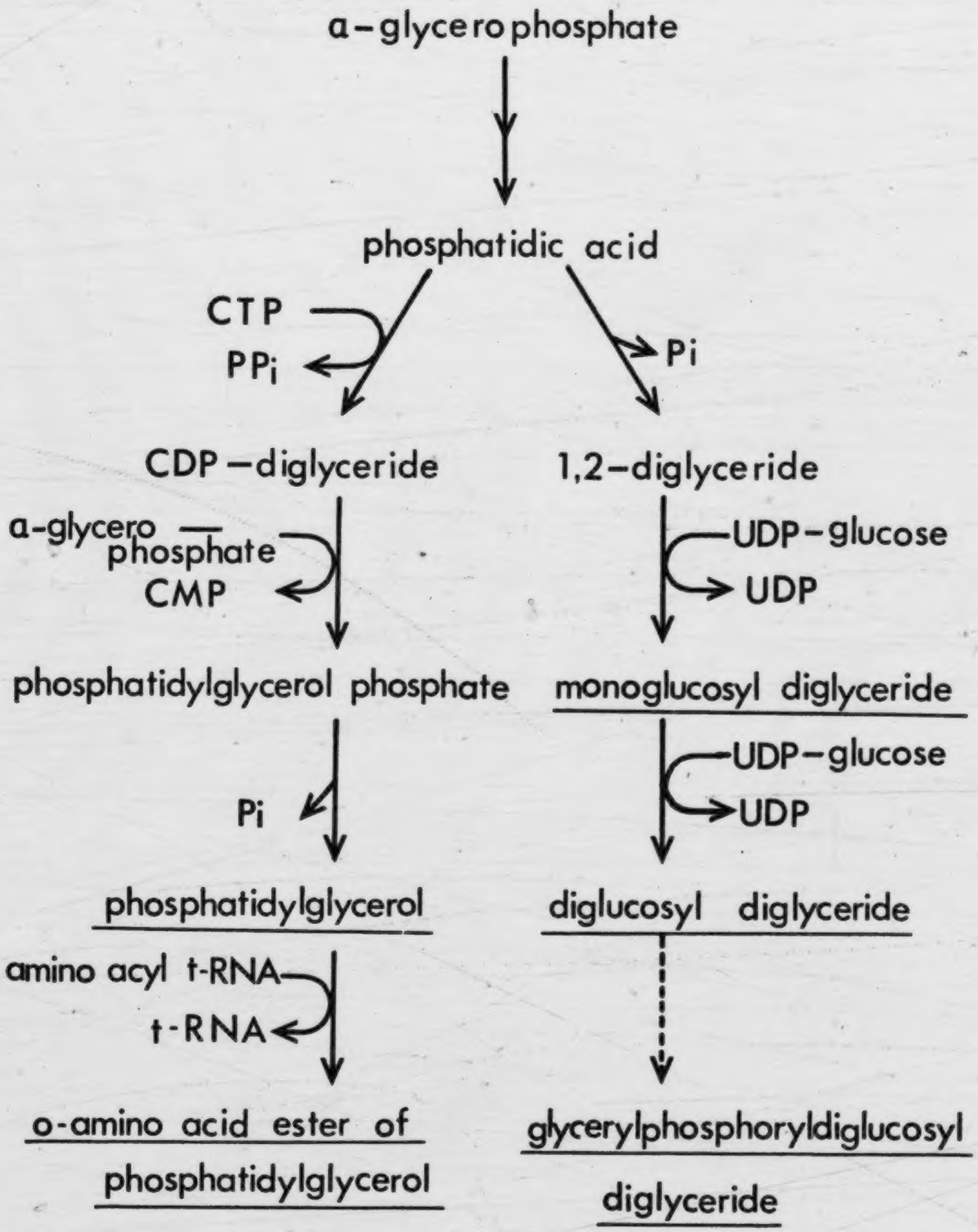
Unlike most other microorganisms, the cell membrane of A. laidlawii B does not contain any conventional nitrogen-containing phospholipids. In fact the three glycolipids (MGDG, DGDG and GPDGDG) comprise about 55 to 60 per cent of the total lipids (Smith, 1968). The rest, about 35 per cent, is comprised by two phospholipids, PG and O-PG, with the former predominating.

Phosphatidic acid (PA) is believed to play a central role in the overall lipid metabolism in A. laidlawii B as well as in bacteria (Lennarz, 1970). PA presumably reacts with cytidinediphosphate, derived from cytidine triphosphate (CTP), to form CDP-DG (cytidine-diphospho-diglyceride), which in turn serves as a precursor for phospholipid biosynthesis (Carter and Kennedy, 1966; Carter, 1968). PA would also be dephosphorylated to give 1,2-diglycerides, which are the precursors for glycolipid synthesis.

Smith (1969a) has shown that A. laidlawii B membrane contains an enzyme or enzymes catalyzing the transfer of glucose from UDPG (uridine-5'-diphosphoglucose) to 1,2-diglycerides to form MGDG, which in turn accepts another molecule of glucose from UDPG to form DGDG. How GPDGDG is synthesized in this organism has not been clearly elucidated as yet, but it probably is synthesized from DGDG, in analogy to the pathway found in Streptococcus faecalis (Pieringer, 1968; Ambron and Pieringer, 1971). The pathway for the biosynthesis of PG has not been examined in this organism, but is presumed to be the same as that demonstrated for bacteria (Chang and Kennedy, 1967a, 1967b); phospholipids are probably synthesized by the transfer of diglyceride from CDP-diglyceride to alpha-glycerophosphate followed by dephosphorylation of the terminal phosphate group. Since Macfarlane (1962) found O-PG in Clostridium welchii, other bacteria also have been reported to contain this rather exotic lipid (Macfarlane, 1964). Koostra and Smith (1969) have reported that A. laidlawii B membranes can catalyze the transfer of an amino acid from an amino acyl-tRNA to PG to form O-PG. Utilising this and other information, the pathway for lipid biosynthesis in A. laidlawii B can be outlined as in Fig. 19.

Since the fatty acyl groups in the membrane lipids of A. laidlawii B are known to be metabolically stable (McElhaney and Tourtellotte, 1970a), any fatty acid compositional differences among lipid classes in this organism have to be generated during lipid biosynthesis, not by deacylation-reacylation reactions. When grown in the absence of exogenous fatty acids, the fatty acid compositions of the two neutral glycolipids are very similar, as are the compositions of the two phospholipids. In addition, each class of lipid, the neutral glycolipids, phospholipids, and phosphorylated glycolipids, exhibits a distinct and different fatty acid spectrum. This would indicate that specific PA species are selectively used for CDP-diglyceride and 1,2-diglyceride synthesis from a common PA pool, or that there are three different PA pools. The latter possibility is perhaps the less likely, since all the enzymes involved in complex lipid biosynthesis in this organism are membrane-bound and the existence of cellular compartments in the absence of intracellular membranes is difficult to imagine. However, these differences in fatty acid composition between the three lipid classes disappear in the presence of some

Fig. 19. The biochemical pathway for the biosynthesis of five major membrane polar lipids of A. laidlawii B.


α-glycerophosphate
phosphatidic acid
CTP
PPi
Pi
CDP-diglyceride
1,2-diglyceride
α-glycero-phosphate
CMP
UDP-glucose
UDP
phosphatidylglycerol phosphate
monoglucosyl diglyceride
Pi
UDP-glucose
UDP
phosphatidylglycerol
diglucosyl diglyceride
amino acyl t-RNA
t-RNA
o-amino acid ester of phosphatidylglycerol
glycerylphosphoryldiglucosyl diglyceride

exogenous fatty acids. Since this organism does not always maintain the fatty acid compositional differences between its neutral glycolipids and phospholipids, the difference in composition between these lipids sometimes noted is probably not biologically significant. At any rate, even the differences in fatty acid composition between neutral glycolipids and phospholipids observed under certain conditions are not as striking in A. laidlawii B as they are in plant and some bacterial systems (Van Deenen, 1966). It is interesting to note that GPDGDG maintains in all cases a somewhat unique fatty acid composition different from DGDG, from which it is presumably synthesized (Pieringer, 1968; Ambron and Pieringer, 1971). The GPDGDG always contains the smallest amount of exogenous fatty acid and the highest quantity of *de novo* synthesized 12:0. This peculiar fatty acid composition of GPDGDG has also been reported by DeKruyff *et al.* (1973). These investigators have also shown by calorimetric studies that the gel to liquid-crystalline phase transition temperature of GPDGDG is almost identical to that of PG, despite the big difference in fatty acid composition between these lipids, although the energy content of the transition of the latter is greater than that of the former.

The question of why certain organisms have certain kinds of polar lipids in their cell membranes (Van Deenen, 1966) remains to be answered. Why some organisms like E. coli have such simple lipid compositions (70 to 80 per cent of lipids being phosphatidylethanolamine) and why other organisms such as A. laidlawii B have much more complex lipid compositions is not understood at present. This diversity of polar lipids has led some investigators to attempt to classify bacteria according to the kind of lipid classes that they contain (Kates, 1964). Recently, studies of the effect of the variations in the structure of apolar fatty acyl chains on the physical state and biological function of membranes have been accelerated by the use of various sophisticated physical methods, but investigations of the function of the membrane lipid polar head groups remain rather preliminary. However, polar head groups in general are considered to be responsible at least for holding together the lipid-protein framework of biomembranes (Van Deenen, 1966). It has also been suggested that polar head groups have some effect on the mobility of apolar fatty acyl chains of the membrane core, thereby affecting the membrane lipid fluidity (Shimshick and McConnell, 1973). It has always been tempting to speculate about some special biological functions, other than the mere structural ones, for the various lipids in the biomembranes, such as in the active transports of nutrients in membrane-associated enzymatic reactions (Lennarz, 1966). Smith (1969b) has observed by pulse-chase experiments with A. laidlawii B that the glucose moiety of carotenol glucoside and GPDGDG, and the phosphate moiety of GPDGDG are actively metabolized, and that the other membrane lipids are metabolically stable. From this observation he has postulated that these two lipid classes, carotenol glucoside and GPDGDG, are specifically involved in glucose transport from the outside to the inside of the cells. However, McElhaney and Tourtellotte (1970a) were unable to observe any turnover of the moieties of any of the membrane lipids in this organism during conditions of normal growth. These conflicting observations are probably due to a difference in the experimental conditions. Since Smith performed his experiments in a buffer system in which the cells were energy-starved and not capable of growth, McElhaney and Tourtellotte have claimed that the labeled GPDGDG observed in Smith's experiment probably exists as a non-physiological metabolic pool which is susceptible to degradation during subsequent metabolism or growth.

5

CHAPTER III

THE CHAIN ELONGATION OF THE EXOGENOUS FATTY ACIDS

Introduction

Animal cells are known to contain two fatty acid chain elongation systems, both of which are distinctly different from the *de novo* fatty acid biosynthetic system. Some microorganisms also seem to have a chain elongation system which is separate from the *de novo* synthetic machinery, but others do not. *A. laidlawii* B is capable of extensively incorporating exogenous fatty acids from the growth medium into the various membrane lipids. This organism has also been reported to be able to elongate exogenous 9-16:1c to 11-18:1c (Romijn *et al.*, 1972). In this chapter, the ability of *A. laidlawii* B to elongate a wide variety of exogenous fatty acids will be determined in order to delineate the specificity of the chain elongation system apparently present in this organism. The effect of varying the concentration of the exogenous fatty acid on the degree of chain elongation will also be determined, and the effect, if any, of the presence of one exogenous fatty acid on the extent of chain elongation of a second exogenous fatty acid will also be studied. Finally, the effect of exogenous cholesterol and variations in the growth temperature on this enzyme system will be investigated in order to gain some insight into the mechanism by which the chain elongation system of *A. laidlawii* B is regulated. I will discuss the possible biological significance of the existence of the fatty acid chain elongation system in this organism and present evidence that this elongation system is distinct from the fatty acid *de novo* biosynthetic system.

A. The effect of the concentration of exogenous fatty acid on the extent of chain elongation

1. Materials and Methods

Ethanolic solutions containing various fatty acids were added to 125 ml of lipid-poor growth medium before inoculation to a final concentration of 0.01 to 0.12 mM. Cells were harvested in late-log or early-stationary phase after 18 to 26 hours of growth. Lipids were extracted and methyl esters of component fatty acids were prepared by the conventional procedure. The fatty acid compositions were determined by GLC. The extent of elongation is obtained by the following equations:

$$\text{First elongation} = \frac{\text{total concentration of all elongation products} \times 100}{\text{concentration of the exogenous fatty acids} + \text{total concentration of all elongation products}}$$

$$\text{Second elongation} = \frac{\text{concentration of elongation products containing four or more additional carbon atoms} \times 100}{\text{total concentration of all elongation products}}$$

2. Results

In the preceding chapter of this dissertation, I pointed out that certain short- and intermediate-chain length exogenous fatty acids can be elongated by A. laidlawii B to their longer-chain homologues, while most long-chain fatty acids do not undergo this chain elongation reaction. This phenomenon suggested to me that this organism may utilize the chain elongation system as another mechanism to regulate membrane lipid fluidity. In this section, I determined the effect of the concentration of the exogenous fatty acid on its degree of chain elongation, before comparing the extent of chain elongation of an exhaustive variety of exogenous fatty acids which may be incorporated to different degrees. For this purpose five different fatty acids are used as the representatives. They are 12:0i, 13:0ai, 9-14:1c, 9-16:1c and 9,12,15-18:3c,c,c. All of them are quite highly elongated, and easily distinguishable on GLC from the acids derived from de novo fatty acid biosynthesis. The results of experiments designed to determine the effect of the concentration of these exogenous fatty acids on the degree of chain elongation are presented in Tables 13 and 14. When 12:0i or 13:0ai is the exogenous fatty acid, the degree of elongation is remarkably constant regardless of the amount of fatty acid which is present in the growth medium or which is incorporated into the membrane lipids. When 9-14:1c is added in the medium, the degree of elongation is again very constant when the amount incorporated varies from 20 to 65 mole per cent. In this experiment 18:1c is omitted from the calculation of the total fatty acid composition. This was done because some 9-18:1c is incorporated into membrane lipids from the residual 9-18:1c present in the defatted growth medium, and this exogenous 18:1c will not be differentiated from the small amount of 9-14:1c elongation product, 13-18:1c, by the GLC analysis. When 9-16:1c is added as the exogenous fatty acid, the extent of elongation is again quite constant when the amount of the acid incorporated varies from 35 to 75 mole per cent. When 9,12,15-18:3c,c,c is used as an exogenous acid, the extent of the chain elongation is again fairly constant regardless of the amount incorporated, from 10 to 40 mole per cent. These experiments clearly demonstrate that the extent of

Table 13. The effect of variations in the concentration of exogenous branched-chain fatty acids on their extent of elongation by A. laidlawii B

Exogenous fatty acid	Amount incorporated (mole %)	Extent of first elongation (%)	Extent of Second elongation (%)
12:0i	18.9	94.1[a]	3.5[a]
	30.0	94.6	4.7
	35.2	90.2	6.8
	42.9	90.3	7.8
	51.0	88.2	10.1
	60.2	89.6	14.1
13:0ai	8.7	83.9	-
	19.7	85.7	7.4
	30.7	87.1	8.3
	45.2	83.3	9.5
	53.2	84.1	10.2
	61.3	83.5	9.9
	68.1	80.7	9.5
	74.2	81.5	9.9

[a] The values listed here for the extent of chain elongation of these branched-chain fatty acids are a little larger than comparable values to be presented in the next section of this chapter. This is because the data presented here was collected at an earlier date, and the activity of the chain elongation system of this organism showed a progressive increase over the time period encompassed by these studies.

Table 14. The effect of variations in the concentration of several exogenous unsaturated fatty acids on their extent of chain elongation by *A. laidlawii* B

Exogenous acid	Amount incorporated (mole %)	Proportion elongated (%)
9-14:1c	22.0	76.8[a]
	37.8	78.0
	41.4	78.3
	55.2	77.4
	61.9	74.3
	65.6	70.3
9-16:1c	36.6	64.4
	54.5	77.7
	65.6	72.7
	68.5	73.5
	75.7	69.6
9,12,15-18:3c,c,c	9.6	36.7
	28.1	34.6
	35.4	30.5
	42.7	35.7

[a] The values listed here for the extent of chain elongation of these unsaturated fatty acids are a little larger than comparable values to be presented in the next section of this chapter. This is because the data presented here was collected at an earlier date, and the activity of the chain elongation system of this organism showed a progressive increase over the time period encompassed by these studies.

chain elongation of exogenous fatty acids depends very little if at all upon the amount of that acid incorporated into the membrane lipids.

3. Discussion

It is conclusively demonstrated in these experiments that the degree of the chain elongation of exogenous fatty acids is fairly constant regardless of the amount of that fatty acid incorporated. However, it is of interest to notice the changes in the pattern of the de novo synthesized fatty acids when the amount of exogenous fatty acid incorporated varies over such a wide range. Particularly for the exogenous mono- and poly-unsaturated fatty acids, as the degree of incorporation increases the spectrum of fatty acids produced by the de novo pathway shifts toward the longer chain length. This can be clearly seen in Table 15, where the preponderance of, for example, endogenous 16:0 over 14:0 increases markedly as the levels of incorporation of the exogenous unsaturates rise. The iso- and anteiso-branched exogenous fatty acids, which have higher melting points than do the unsaturated fatty acids used in this study, do not induce such a marked shift in the average chain length of the endogenous fatty acids. One can interpret this increased production of longer-chain, high-melting endogenous fatty acids as a compensatory response to the utilization of the lower-melting exogenous fatty acids and their elongation products. This organism might have been expected to increase the degree of chain elongation and maintain the de novo synthetic pattern constant instead, in response to the incorporation of larger amounts of exogenous low-melting fatty acids. This response would also compensate for the effect of exogenous acid in progressively

Table 15. The ratio of 16:0 to 14:0 in the total membrane lipid of *A. laidlawii* B and the amount of exogenous branched-chain or unsaturated fatty acid incorporated into the membrane lipid

Exogenous fatty acid	Ratio of 16:0 to 14:0	
	lowest incorporation	highest incorporation
12:0i	1.13	1.21
13:0ai	1.57	1.64
9-14:1c	1.50	1.91
9-16:1c	1.71	4.40
9,12,15-18:3c,c,c	1.66	6.93

increasing the fluidity of the membrane lipids. However, _A. laidlawii_ B maintains the degree of chain elongation of exogenous fatty acids constant, and changes only the _de novo_ synthetic pattern in response to the amount and nature of the incorporated exogenous fatty acid. This result may suggest that the _de novo_ fatty acid biosynthetic system and the chain elongation system are different from each other, and that the degree of chain elongation is regulated only by the intrinsic properties of its fatty acid substrate, as will be discussed later in this chapter.

B. The effect of the structure of exogenous fatty acids on the extent of chain elongation

1. Materials and Methods

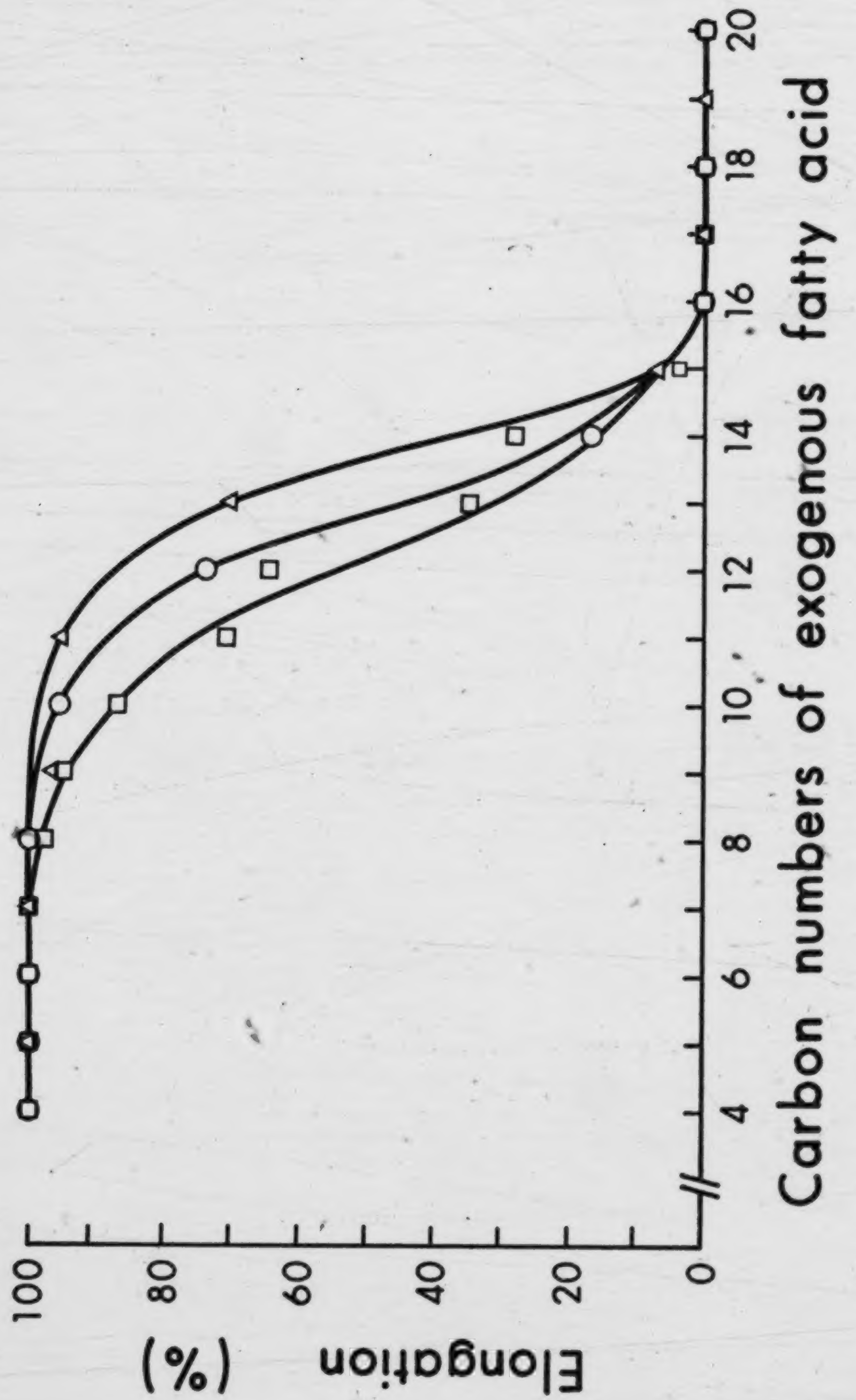
Ethanolic solutions containing various fatty acids were added to 125 ml of lipid-poor growth medium before inoculation to give a final fatty acid concentration of 0.12 mM. _A. laidlawii_ B cells were harvested in late-log or early-stationary phase after 16 to 20 hours of growth. Lipids were extracted and methyl esters were prepared either by the small scale procedure or by the conventional procedure, depending on the chain length of the exogenous fatty acid, as was stated in the preceding chapter. The fatty acid composition was determined by GLC. The extent of chain elongation was calculated as before.

Since this organism can synthesize _de novo_ fair amounts of 12:0, 14:0 and 16:0 even in the presence of exogenous fatty acids, fatty acids incorporated directly from the media or elongated from the exogenous short-chain, even-numbered fatty acids have to be distinguished from those synthesized _de novo_ from acetate. For this purpose radioactive precursors were used. Radioactive fatty acids or their sodium salts were mixed with cold carrier acids to bring the final concentration to 0.12 mM. The following are the radioactive precursors used: sodium butyrate-1-C^{14} (50 μC), sodium-hexanoate-1-C^{14} (50 μC), octanoic acid-1-C^{14} (25 μC), decanoic acid-1-C^{14} (25 μC), dodecanoic acid-1-C^{14} (25 μC), tetradecanoic acid-1-C^{14} (25 μC), and hexadecanoic acid-1-C^{14} (25 μC). Cells were grown and the total membrane lipid extracted as before. The fatty acid composition and the distribution of radioactivity were simultaneously analyzed by the RAM-GLC system. The extent of chain elongation was calculated as before, except that the areas from the radioactivity measurements were used to quantitate the amounts of the appropriate exogenous fatty acids present, instead of using the data from the GLC analysis, as in previous experiments when nonradioactive exogenous fatty acids were being utilised.

2. Results

As was briefly discussed in the preceding chapter of this dissertation, short-chain exogenous acids are exhaustively elongated, but the degree of chain elongation progressively decreases with an increase in the carbon number of the fatty acid. Fig. 20 illustrates the effect of the chain length on the extent of the first elongation of various straight-chain, iso- and anteiso-branched exogenous saturated fatty acids. This figure also depicts the effect of the introduction of methyl-branching into straight-chain fatty acids on the extent of chain elongation. The introduction of isobranching results in more extensive elongation of the longer-chain substrate while anteisobranching promotes the extent of elongation still further. Exogenous short-chain saturated fatty acids undergo essentially complete

Fig. 20. The effect of the chain length and chemical structure of an exogenous fatty acid on the extent of the first chain elongation of that fatty acid by A. laidlawii B. Cells were grown at 34°C in the presence of a single exogenous fatty acid at a final concentration of 0.12 mM. The total membrane lipids were extracted from late-log to early-stationary phase cells as described in the text. The fatty acid composition was determined by GLC, and the extent of the first chain elongation (%) was calculated by the method described in the text. -□-□-, -○-○- and -△-△- represent straight-chain saturated, isobranched and anteisobranched fatty acids, respectively. The values presented here are averages of at least triplicate experiments.


Elongation (%)
100
80
60
40
20
0
4
6
8
10
12
14
16
18
20
Carbon numbers of exogenous fatty acid

conversion to their longer chain homologues until the carbon number reaches 10 or 11, at which point a small amount of 10:0 or 11:0 is incorporated directly, although most continues to be elongated to 12:0, 13:0, 14:0, 15:0 and 16:0. When the chain length of the exogenous saturated fatty acid reaches 16 carbon atoms, chain elongation ceases. The minimum chain length for the isobranched and anteisobranched exogenous acids, which produces less than quantitative elongation is higher, being 12 and 13 carbon atoms, respectively. In addition, the average chain length of the products of chain elongation was also shifted upward upon the introduction of a methyl-branch. Again, chain elongation ceases when the carbon number of the isobranched and anteisobranched fatty acid reaches 16 and 17, respectively.

The effect of the presence of cis- or trans-double bonds or a cyclopropane ring in the exogenous fatty acid on the extent of chain elongation is presented in Table 16. If the extent of chain elongation is compared within a class of fatty acids having the same functional group, the following orders are obtained:

Straight chain 4:0-7:0 > 8:0 > 9:0 > 10:0 > 11:0 > 12:0
> 13:0 > 14:0 > 15:0-20:0

Isobranched 4:0i-10:0i > 12:0i > 14:0i > 16:0i , 18:0i

Anteisobranched 5:0ai-11:0ai > 13:0ai > 15:0ai > 17:0ai ,
19:0ai

Table 16. The chain elongation of various exogenous unsaturated and cyclopropane fatty acids by A. laidlawii B

Exogenous fatty acid	Extent of First elongation (%)	Extent of Second elongation (%)
cis-monounsaturated		
9-14:1c	60.6	11.2
9-16:1c	48.2	0
9-18:1c	4.0	0
11-20:1c	0	0
13-22:1c	0	0
15-24:1c	0	0
trans-monounsaturated		
9-14:1t	23.5	20.8[a]
9-16:1t	1.1	0
9-18:1t	0	0
cis-polyunsaturated		
9,12-18:2c,c	16.9	0
9,12,15-18:3c,c,c	35.5	0
5,8,11,14-20:4c,c,c,c	0	0
cyclopropane		
9,10-17:0cp,c	12.5	0
9,10-19:0cp,c	0	0
9,10-19:0cp,t	0	0
straight-chain		
14:0	28.8	0
16:0	0	0
18:0	0	0
20:0	0	0

[a] The value reported here is probably much higher than the real value for the second chain elongation of 9-14:1t, since the 13-18:1t derived from 9-14:1t can not be differentiated by conventional GLC from the 9-18:1c which is incorporated from the lipid-poor growth medium.

The values presented in this table are the averages of at least triplicate experiments.

Monounsaturated > 9-14:1c > 9-16:1c > 9-14:1t > 9-18:1c
> 9-16:1t > 9-18:1t, 11-20:1c, 13-22:1c,
15-24:1c

Polyunsaturated 9,12,15-18:3c,c,c > 9,12-18:2c,c
> 5,8,11,14-20:4c,c,c,c

Cyclopropane 9,10-17:0cp,c > 9,10-19:0cp,c , 9,10-19:0cp,t

From the above observation it is quite reasonable to conclude that the shorter the chain length, the greater the extent of elongation among exogenous fatty acids containing the same chemical functional group.

Finally, the effect of the introduction of various functional groups into an exogenous saturated fatty acid on the extent of chain elongation is shown in Table 17. When the chain length is 12, the introduction of both iso- and anteiso-branching slightly increases the extent of chain elongation. When the chain length is 14, the introduction of iso- and anteiso-branching, or a trans-double bond slightly reduces the extent of chain elongation, whereas the introduction of a cis-double bond greatly enhances the chain elongation. When the chain length is 16, the introduction of both cyclopropane and cis-double bonds increase the extent of chain elongation, whereas the presence of a trans-double bond or a methyl branch does not have much effect. When the chain length is 18, the introduction of isobranching, trans-unsaturations do not enhance the chain elongation. However, the introduction of more than one cis-double bond greatly increases the extent of elongation.

Table 17. The effect of the introduction of various functional groups into an exogenous straight-chain, saturated fatty acid on the extent of chain elongation of that fatty acid by *A. laidlawii* B

Exogenous fatty acids	Number of carbon atoms in the fatty acid molecule				
	12 (%)	14 (%)	16 (%)	18 (%)	20 (%)
straight-chain	67.1	28.8	0	0	0
isobranched	74.2	16.8	0	0	0
anteisobranched[a]	70.7	8.2	0	0	0
cis-monounsaturated	-	60.6	48.2	4.0	0
trans-monounsaturated	-	23.5	1.1	0	0
cis-polyunsaturated	-	-	-	16.9[b]	0[c]
				35.5[d]	
cyclopropane	-	-	12.5[e]	0[f]	-
				0[g]	

[a] The total number of carbon atoms in the exogenous anteisobranched fatty acids is greater by one than is indicated in the table.

[b] 9,12-18:2c,c

[c] 5,8,11,14-20:4c,c,c,c

[d] 9,12,15-18:3c,c,c

[e] 9,10-17:0cp,c

[f] 9,10-19:0cp,c

[g] 9,10-19:0cp,t

The values presented in this table are the averages of at least triplicate experiments.

3. Discussion

A. laidlawii B is clearly able to elongate many exogenous fatty acids which have melting points too low to permit direct incorporation into the membrane lipids. By the addition of the appropriate number of two-carbon units, these lower-melting fatty acids are converted to their higher-melting analogues, which are then utilized in the biosynthesis of membrane lipids which now have a more optimum degree of fluidity. Furthermore, this organism seems to be able to regulate the extent of chain elongation depending upon the nature of the exogenous fatty acids. It is clearly shown in Fig. 20 and Table 16 that the extent of elongation is dependent upon the chain length. The shorter the chain length of a fatty acid in a given chemical class, the greater is the extent of chain elongation. Table 17 also demonstrates the effect of the introduction of various chemical groups into a straight-chain saturated acid on the extent of chain elongation. Considering a series of fatty acids having the same chain length, the introduction of a methyl branch, a *trans*-double bond, a *cis*-cyclopropane ring, a *cis*-double bond, and two *cis*-double bonds progressively increase the extent of chain elongation in that order. Interestingly enough, the effectiveness of these chemical groups in promoting chain elongation roughly parallels their effectiveness in disrupting the close packing of the lipid hydrocarbon chains in a lipid bilayer. We can conclude from these results that, in general, the lower the melting point of an exogenous fatty acid, the greater is the extent of elongation and the longer is the chain length of the derivatives produced. However, this rule does not strictly hold when the extent of chain elongation of certain cis-, mono- and poly-unsaturated fatty acids are examined. For example, 5,8,11,14-20:4, which has four cis-double bonds and thus a very low melting point, is not elongated at all. Also, we might have expected that 9-18:1c would undergo quite extensive chain elongation because of its relatively low melting point, but it is elongated only a little. It seems, therefore, that there is another specificity for the elongation system, an intrinsic chain-length specificity, superimposed over the physicochemical specificity just discussed; shorter-chain fatty acids are elongated in preference to the longer-chain fatty acids despite the introduction of very disruptive chemical groups on certain longer-chain fatty acids. This lack of elongation of longer-chain but lower-melting fatty acids appears to be compensated for by a shift of the pattern of de novo synthesis, as was discussed in the preceding chapter of this dissertation. This compensatory shift in the average chain length is demonstrated in Table 18, where the fatty acid composition of the total lipids from cells grown with 11:0 or 9-18:1c is presented. When 11:0 is present in the growth medium, only 23.3 per cent is directly incorporated into the membrane lipids, and 76.7 per cent of the 11:0 taken up is elongated into its longer homologues, 13:0, 15:0 and 17:0, prior to its biosynthetic utilization; 11:0 itself and these longer homologues altogether comprise about 63 mole per cent of the total esterified fatty acids. The ratio among de novo synthesized 12:0, 14:0 and 16:0 is 1.00:2.77:2.47. When 9-18:1c is the exogenous fatty acid, only 4 per cent is elongated and comprises about 70 mole per cent of the total esterified fatty acids. However, the ratio among de novo

Table 18. The effect of the incorporation of exogenous 11:0 or 9-18:1c on the fatty acid composition of the total membrane lipids of A. laidlawii B

Fatty acids found	Exogenous fatty acids	
	11:0 (mole %)	9-18:1c (mole %)
11:0	15.2[a]	-[a]
12:0	5.3	3.3
13:0	24.5	1.4
14:0	14.7	7.2
15:0	21.2	1.4
16:0	13.1	16.9
17:0	2.0	0
18:0	2.8	0
9-18:1c	1.0	67.2
11-20:1c	-	2.7

[a] These values are representative of at least triplicate experiments.

synthesized 12:0, 14:0 and 16:0 is 1.00:2.18:5.12. It is clear that the lack of elongation of 9-18:1c is compensated for by the preferential synthesis of a longer-chain acid, 16:0. These results can again be interpreted as a successful attempt by the cells to maintain the fluidity of the hydrocarbon core of the membrane within a certain range. The mechanism by which this chain elongation system regulates the extent of elongation of an exogenous fatty acid substrate is not understood as yet. However, the specificity of this system is clearly not based on any specific chemical properties of the exogenous fatty acids, because there is no apparent relationship between the degree of elongation and the presence of any particular functional group, and also because the extent of chain elongation is dependent on the chain length of fatty acids belonging to the same chemical class. The chain elongation system seems to regulate the elongation rather by a physical property, namely the potential disruptive effect of the acids on the membrane hydrocarbon core when incorporated, as well as by the intrinsic specificity of the system for the chain length of the exogenous acids. This is further substantiated by the fact that this system can differentiate the positional isomers of monounsaturated fatty acids. In the case of monounsaturated fatty acids, the position of the double bond should have an effect on the potential disruptive effect of the bond when these monounsaturated acids are incorporated into the membrane lipids. Fatty acids having a double bond close to the methyl end of the molecule have higher melting points than fatty acids having the double bond close to the middle of the chain (Gunstone, 1967). The effect of double bond position on the extent of chain elongation is clearly seen if a comparison is made between the second elongation from 9-14:1c, the conversion of 11-16:1c to 13-18:1c, and the first elongation from 9-16:1c to 11-18:1c. About 50 per cent of 9-16:1c is elongated to 11-18:1c, but only 10 per cent of 11-16:1c is elongated to 13-18:1c. In fact in any other cases where chain elongation does not affect the relative location of the functional group, these differences are not observed at all. For example, the extent of the second elongation from 12:0i, the conversion of 14:0i to 16:0i, and that of the first elongation from 14:0i to 16:0i, are very close. The extent of the second elongation from 13:0ai, the conversion of 15:0ai to 17:0ai, and that of the first elongation from 15:0ai to 17:0ai are also very similar.

Although the amounts of various exogenous fatty acids incorporated are not the same in each case, this should not affect the interpretation of these results, since it has been clearly demonstrated in the preceding section of this chapter that the degree of chain elongation is not very dependent on the amounts of exogenous fatty acid incorporated. The difference in the degree of chain elongation observed here, therefore, can be attributed solely to the nature of the exogenous fatty acids.

The exact mechanism by which the chain elongation takes place in _A_. _laidlawii_ B is not known. Animal cells contain two different elongation pathways other than the _de_ _novo_ fatty acid synthetic pathway; one is located in the microsomes and the other in the mitochondria, in contrast to the fatty acid synthetase complex and acetyl-CoA carboxylase, which are in the cytosol (Wakil, 1970). Microsomal and mithochondrial systems use malonyl- and acetyl-CoA, respectively, for the chain elongation. Some difference of cofactor or cofactors required for the reduction steps are also suggested. Although there is some disagreement over whether CoA or ACP is the preferred coenzyme for the microsomal elongation system, only acetyl-CoA can serve as two-carbon donor in the mitochondrial system (Wakil, 1970). The mitochondrial system is essentially the reverse of the *beta*-oxidation pathway, except that the fatty acyl-CoA dehydrogenase is replaced by an enzyme which catalyzes the reduction of the *trans*-*alpha*, *beta*-unsaturated acyl-CoA by utilization of NADPH, instead of the flavoproteins, as the source of reducing equivalents. Both systems can elongate saturated fatty acids of chain length 10 to 16 carbon atoms, at higher rates than shorter- or longer-chain saturated acids, and both systems elongate unsaturated acids of 16 and 18 carbon atoms faster than the corresponding saturated acids (Nugteren, 1965; Wakil, 1970). *Mycobacterium* *phlei* is also known to have a chain elongation system as well as a system for *de* *novo* fatty acid synthesis (Brindley *et* *al*., 1969; Matsumura *et* *al*., 1970). This chain elongation system is dissociable into its component enzymes like plant and bacterial fatty acid synthetases, and specifically uses 16:0-CoA or 18:0-CoA but not 8:0-CoA, for chain initiation. However, the *de* *novo* system is a typical non-dissociable, multi-enzyme complex like yeast and animal fatty acid synthetases (Kumer *et* *al*., 1972), and uses acetyl-CoA as the primer. *Lactobacillus* *plantarum* also appears to elongate some exogenous fatty acids (Weeks and Wakil, 1970). Interestingly enough *E*. *coli* does not seem to have any activity for chain elongation despite the active *de novo* fatty acid biosynthetic pathway and appreciable *beta*-oxidation activity. Weeks and Wakil (1970) have speculated that the difference between *L. plantarum* and *E. coli* might possibly be explained if only the former has a long chain acyl-CoA-ACP transacylase enzyme, capable of transferring the acyl group of the exogenous fatty acid from CoA to ACP; these investigators assumed of course that the ACP derivative, but not the CoA derivative, can participate in chain elongation. However, Alberts *et al.* (1972) have shown that *beta*-ketoacyl-ACP synthetase from *E. coli* can catalyze the fatty acyl-CoA-ACP acyltransfer reaction as well as the condensing reaction, although it is not known whether this transfer reaction also takes place *in vivo* or not. In contrast to *E. coli*, a fatty acid synthetase mutant of *Saccharomyces cerevisiae*, which lacks only the condensing activity in the fatty acid synthetase complex (Schweizer and Bolling, 1970) is able to elongate some exogenous fatty acids in spite of its inability to synthesize fatty acids *de novo* from acetate (Orme *et al.*, 1972). This yeast mutant is a so-called "petite" mutant and has no functional mitochondria. It is therefore unlikely that this elongation is accomplished by the mitochondrial elongation system seen in animal cells. The existence of a microsomal chain elongation system has never been demonstrated in *S. cerevisiae*. This may suggest that there is an independent "chain elongation" system in this organism different from the *de novo* synthetic system. However, there is a possibility that there are two different condensing enzymes in wild type cells, one being specific for short-chain and the other for intermediate-chain fatty acids. This mutant may have only the condensing enzyme which can elongate exogenous intermediate-chain fatty acids. Recently, wild type baker's yeast fatty acid synthetase has been reported to be able to use both acetyl-CoA, and 9:0- and 10:0-CoA as "primers". However, neither 4:0- or 6:0-CoA, nor 14:0- and 16:0-CoA, are good substrates for the yeast synthetase (Pirson _et_ _al_., 1973). Two non-sterol requiring _Acholeplasma_, _A_. _laidlawii_ A and _A_. sp KHS, are also reported to elongate some exogenous fatty acids (Panos and Rottem, 1970; Panos and Henrikson, 1969). The effect of the position and geometry of the double bond on the degree of chain elongation is also noticed in these strains. Although there certainly are chain elongation systems in animal cells different from the _de_ _novo_ synthesis system, there has not been any conclusive evidence for the existence of distinct chain elongation systems in any prokaryotic microorganism.

It would be of interest to know whether _A_. _laidlawii_ B has an independent chain elongation system different from the _de_ _novo_ fatty acid biosynthetic system, or whether the _de_ _novo_ synthetic system can also elongate exogenous fatty acids. I favor the former alternative because of the following observations: 1. When the total incorporation of straight-chain saturated, iso- and anteiso-branched acids are determined (Fig. 13), the minimum incorporation is not obtained with the shortest chain examined. For example, 4:0 is incorporated to a greater extent than 5:0, 6:0 and 7:0; 5:0ai is incorporated more efficiently than 7:0ai and 9:0ai; 4:01 is more highly incorporated compared to 6:01, 8:01 and 10:01. 2. When varied amounts of the exogenous fatty acids are incorporated, the degree of the chain elongation of the exogenous acids remains constant, although the pattern of the _de novo_ fatty acid biosynthetic system changes quite dramatically. 3. When the exogenous acid is either acetate or 4:0, the ratio of 14:0 to 16:0 derived from these exogenous acids is about 0.7 to 0.9. When the cells are grown in the absence of exogenous acids, the ratio is also about 0.7 to 0.9. When the exogenous acid is one of the following acids (8:0, 10:0, 12:0, 14:0), the ratio of 14:0 to 16:0 derived from these acids is 2.4 to 2.7. In other words the _de novo_ system, utilizing the very short-chain exogenous primers, produces fatty acids with a longer average chain length than does the elongation system, which utilizes exogenous intermediate-chain fatty acids. However, it is still a possibility that some components of _de novo_ fatty acid synthetic machinery are shared by the chain elongation system and further work will be necessary to firmly resolve this question.

C. The effect of the incorporation of various long-chain exogenous fatty acids on the degree of chain elongation of shorter-chain fatty acids simultaneously added to the growth medium

1. Materials and Methods

Ethanolic solutions containing either 13:0 or 13:0ai were added to 125 ml of lipid-poor growth medium before inoculation to a final concentration of 0.06 or 0.12 mM. To other 125 ml volumes of lipid-poor growth media were added mixtures of either 13:0 or 13:0ai and either 16:0 or 9-18:1c, with the final concentration of each fatty acid in the growth medium being 0.06 mM. Cells were harvested in late-log or early-stationary phase after 23 to 26 hours of growth.

The total membrane lipids were extracted and methyl esters of component fatty acids were prepared by the conventional procedure. The fatty acid compositions were determined by GLC. The extent of chain elongation was obtained as before.

2. Results

It is of interest to see how the chain elongation system of A. laidlawii B responds to a situation where an acid, which is elongated when added alone, is added simultaneously into the growth medium with another fatty acid, which is elongated little but whose incorporation into the membrane lipid will either enhance or disrupt the close packing of the complex lipid molecules in the membrane.

The degree of elongation of either 13:0 or 13:0ai was determined in the absence or in the presence of either 16:0 or 9-18:1c. Incorporation of 16:0, a long-chain saturated fatty acid, reduces the fluidity of the membrane lipids (McElhaney, 1974). Incorporation of 9-18:1c, on the other hand, markedly increases the fluidity of the membrane lipids. Instead of 17:0, 16:0 was used in this experiment, because the former would be difficult to separate clearly from the 17:0ai derived from the chain elongation of exogenous 13:0ai. The results of these experiments are presented in Table 19. When A. laidlawii B is grown with 13:0ai (0.06 mM or 0.12 mM) alone, this fatty acid (and its elongation products) accounts for 45.3 mole per cent of the total esterified fatty acids in one case (0.06 mM) and 66.7 mole per cent in the other (0.12 mM). Despite this difference in the amount of 13:0ai incorporated, the degree of the chain elongation of this acid is constant, with about 86 per cent and 13 per cent of 13:0ai

Table 19. The effect of the incorporation of various long-chain exogenous fatty acids on the degree of chain elongation by A. laidlawii B of shorter-chain fatty acids simultaneously added to the growth medium

Exogenous fatty acids	Fatty acid concentration in the total membrane lipids		Extent of elongation of 13:0ai or 13:0	
	16:0 or 9-18:1c (mole %)	13:0ai or 13:0 (mole %)	First (%)	Second (%)
13:0ai alone	-	45.3	87.7	13.4
	-	66.7	85.5	12.0
13:0ai + 16:0	42.1	41.7	49.2	5.9
	46.4	36.1	42.8	4.7
	53.4	27.8	25.9	0
13:0ai + 9-18:1c	57.7	15.5	82.6	38.3
	61.3	11.1	84.7	31.9
13:0 alone	-	80.0	35.0	5.5
13:0 + 16:0	42.3	42.1	20.0	7.4
	45.2	40.4	17.6	5.6
	48.7	35.6	22.2	6.3
13:0 + 9-18:1c	40.4	43.5	44.4	7.2
	46.5	36.9	54.5	9.5
	51.2	29.8	66.1	14.2

being elongated to the 15:0ai and 17:0ai, respectively. In the presence of exogenous 13:0ai, this organism synthesizes mainly 16:0 and 14:0, with the former predominating slightly, and the amount of exogenous 9-18:1c incorporated is less than 5 per cent. Increasing the amount of 16:0 incorporated into the membrane lipids, which is added simultaneously with 13:0ai in the growth medium, results in a decreased degree of chain elongation of 13:0ai. When 16:0 comprises about 50 mole per cent of the total fatty acids in the membrane lipids, only 26 per cent of 13:0ai is elongated to 15:0ai and none to 17:0ai, compared to 86 and 13 per cent, respectively, when 13:0ai is the only exogenous fatty acid added. If 9-18:1c instead of 16:0 is incorporated into the membrane lipids from the growth medium, the degree of the chain elongation of 13:0ai is increased, especially the second elongation, from 15:0ai to 17:0ai. Very similar effects of 16:0 and 9-18:1c on the elongation of 13:0 are also observed.

3. Discussion

In the preceding section of this chapter, it has been demonstrated that both 13:0 and 13:0ai are extensively converted to their longer-chain homologues when they are supplied alone in the growth medium. This result has been interpreted as a successful attempt by A. laidlawii B to maintain the fluidity of the fatty acyl core of the cell membrane by converting these lower-melting exogenous fatty acids to longer-chain derivatives having more optimal physical properties. If this interpretation is correct, then one might predict that A. laidlawii B would be capable of regulating the degree of the chain elongation of these acids in response to the incorporation of other exogenous fatty acids which greatly enhance or inhibit the close-packing of the membrane lipids. For example, if 16:0 is added to the growth medium with either 13:0 or 13:0ai, the degree of the chain elongation of the latter fatty acids is expected to be inhibited by 16:0, because 16:0 will increase the cohesive forces among fatty acyl chains. This is exactly what is found experimentally. Furthermore, it is rather clearly demonstrated in the experiments with 13:0ai that the more 16:0 is incorporated in the membrane lipids, the less 13:0ai is elongated. The simultaneous incorporation of 16:0 will compensate for the potential disruptive effect of the incorporation of 13:0ai, so that it would no longer be necessary for the cells to reduce the disruptive effect of 13:0ai by extensively elongating it. On the other hand, if 9-18:1c is incorporated into the A. laidlawii B cell simultaneously with either 13:0 or 13:0ai, the degree of the chain elongation of the latter acids is expected to be increased by the presence of 9-18:1c, because 9-18:1c will greatly disturb the close-packing of fatty acyl chains in the cell membrane when incorporated (Van Deenen, 1966). This is again exactly what is found experimentally. It is also clearly demonstrated that the more 9-18:1c is incorporated into the membrane lipids, the greater the degree of chain elongation of both 13:0 and 13:0ai. The simultaneous incorporation of 9-18:1c will further enhance the potential disruptive effect of exogenous 13:0 and 13:0ai, and the cells would have to elongate them more extensively to reduce this over-all potential disruptive effect of the incorporation of both pairs of exogenous acids upon the fatty acyl core of the membrane.

These results conclusively show that the degree of the chain elongation of any exogenous fatty acid is not fixed but instead is regulated in a coherent manner in response to the incorporation of other exogenous fatty acids. This finding further strengthens the hypothesis put forth earlier that this organism regulates the degree of chain elongation of exogenous fatty acids according to their physicochemical properties, as well as by the intrinsic chain length specificity of the elongation system, in order to maintain the fluidity of the fatty acyl core of the membrane lipids within a certain range.

The detailed mechanism of how 16:0 and 9-18:1c affect the chain elongation system remains to be elucidated in future investigations. However, it is of interest to recall that these exogenous acids also affect the pattern of de novo fatty acid biosynthesis.

D. The effect of the incorporation of cholesterol and of alterations in the growth temperature on the chain elongation of the exogenous fatty acids

1. Materials and Methods

The effect of cholesterol: Ethanolic solutions of 13:0ai with and without cholesterol (3.1 mg) were added to 125 ml of lipid-poor growth medium before inoculation to a final concentration of fatty acid of 0.12 mM. Cholesterol was the ULTREX grade from Baker Chemical Company (Phillipsburg, New Jersey, U.S.A.). Cells were harvested in late-log or early-stationary phase after 16 hours of growth.

The effect of growth temperature: Cells were grown in 250 ml volumes of lipid-poor growth medium at 5 degree intervals from 20° to 40°C. Cell growth was followed by measuring the change in the absorbancy at 450 nm with time. Cells were again harvested in late-log to early-stationary phase. Lipids were extracted, methyl esters of fatty acids were prepared as described before, and their compositions were analyzed by GLC. The degree of chain elongation of 13:0ai was calculated by the equation given in a previous section of this chapter.

2. Results

In the absence of cholesterol, 90.3 per cent of the incorporated 13:0ai is elongated to 15:0ai, 21.7 per cent of which is in turn elongated to 17:0ai. In the presence of cholesterol, 89.5 per cent of 13:0ai is elongated to 15:0ai, 19.7 per cent of which is further elongated to 17:0ai. The incorporation of cholesterol into the cell membrane therefore affects very little the degree of chain elongation of exogenous 13:0ai. It is of interest to note that the extent of the incorporation of the other exogenous acids is also very little affected by the incorporation of cholesterol.

As illustrated in Table 20, the growth temperature affects only moderately the degree of chain elongation of the exogenous fatty acids. The degree of elongation of 9-14:1c is lower at 20°C than at 25°, 30° and 35°C. The value at 20°C is somewhat doubtful due to the poor growth observed at this temperature. The elongation of 9-14:1t at 40°C is higher than at 35°, 30° and 25°C. However, it is even a little

Table 20. The effect of the growth temperature on the extent of chain elongation of various exogenous fatty acids by *A. laidlawii* B

Exogenous fatty acid	Temperature of growth (°C)				
	40	35	30	25	20
11:0	-	90.2[a]	88.8	90.9	-
12:01	-	84.7	80.7	81.4	68.4
13:0ai	-	74.8	78.7	76.6	80.1
9-14:1c	-	65.3	68.9	68.5	54.3
9-14:1t	47.6	29.8	28.6	31.5	-

[a] All values are given in per cent and are representative values from duplicate experiments.

lower at 35° and 30°C than at 25°C. If these values are all correct the degree of chain elongation of 9-14:1t drops from 25° to 35°C and abruptly increases at 40°C. It is rather difficult to interpret this result, and I am rather doubtful of the value at 40°C. The degree of second and third elongations are also very little affected by the growth temperature.

3. Discussion

McElhaney (1974) has studied the thermal behavior of the membrane lipids of A. laidlawii B and has demonstrated that at the growth temperature, 34°C, lipids in the membrane from the cells grown with 17:0ai exist exclusively in the liquid-crystalline form. In his experiment, 17:0ai comprises about 90 mole per cent of the total membrane lipid fatty acid. In this present experiment, about 90 per cent of the incorporated 13:0ai is elongated to 15:0ai, and about 20 per cent of 15:0ai is further elongated to 17:0ai. These three anteisobranched acids altogether comprise about 70 mole per cent of the total membrane lipid fatty acids. The lipids in this membrane, therefore, are expected to exist exclusively or predominantly in the liquid-crystalline state. Cholesterol incorporated in this membrane (less than 20 mole per cent) is reasonably assumed to interact with liquid-crystalline lipid and reduce the fluidity of the hydrocarbon core, as was discussed in the first chapter of this dissertation.

The simultaneous incorporation of cholesterol might be expected to reduce the degree of the chain elongation of the exogenous acid, 13:0ai, in analogy to the effect of 16:0, since both compounds reduce the disruptive effect of the incorporation of lower-melting fatty acids. However, the incorporated cholesterol has no effect at all on the degree of chain elongation. Therefore, 16:0 probably reduces the degree of the chain elongation of the exogenous fatty acids through a direct interaction with the chain elongation enzyme system, as opposed to exerting its effect indirectly via a more complex regulatory mechanism which can sense the physical state of the membrane lipids.

The degree of chain elongation of exogenous fatty acids by *A. laidlawii* B remains constant regardless of the growth temperature, from 20° to 40°C. In fact, this organism does not grow normally below 20°C or above 40°C, and the maximum cell density as well as the growth rate are highly dependent upon the growth temperature (McElhaney, 1974). This narrow range of permissible growth temperatures may be due to the lack of the ability of this organism to significantly change the pattern of fatty acid *de novo* synthesis or the degree of chain elongation of exogenous fatty acids. Again, if the chain elongation system regulates the degree of elongation only in response to a change in the physical state of the membrane lipids, increasing the environmental temperature would be expected to increase the degree of chain elongation, which is not observed. It is tempting for me to speculate that the enzyme system responsible for the chain elongation has an intrinsic specificity for the physicochemical properties and the chain length of the exogenous fatty acid, and that it is this specificity which is directly affected by the presence of other exogenous fatty acids.

CHAPTER IV

THE POSITIONAL SPECIFICITY OF FATTY ACIDS

Introduction

Non-random distributions of fatty acyl groups in various phospholipids are almost universally observed; with a very few exceptions, saturated and unsaturated fatty acids are preferentially esterified at the 1-position and 2-position, respectively, of the glycerol moiety (Van Deenen, 1966). McElhaney and Tourtellotte (1970b) have systematically studied the positional specificity of a number of exogenous fatty acids in _A_. _laidlawii_ B phosphatidylglycerol by successfully taking advantage of the fact that the fatty acid composition of this organism can be manipulated with ease. These investigators have confirmed and extended previous observations on the asymmetric positional distribution of esterified fatty acids and elucidated the relationship between the structure and chain length of a fatty acid and its positional specificity. In fact, to the best of my knowledge, this is the only extensive and systematic study of fatty acid positional specificity in phospholipids thus far reported. The cell membrane of _A_. _laidlawii_ B, however, contains glycolipids as well as phospholipids, and in fact the glycolipids are quantitatively predominant. The positional specificity of fatty acyl groups in the glycolipids in this organism, however, have never been studied.

Most plants contain glycolipids, and the positional specificity of a limited number of fatty acids in some of these glycolipids have been studied. However, conflicting results have been reported with the various plant glycolipids in contrast to the rather universal results obtained with a variety of different phospholipids from diverse sources. Noda and Fujiwara (1967) reported that the less-highly unsaturated acids were esterified mainly at the 1-position and the more-highly unsaturated acids at the 2-position of glycerol in monogalactosyl and digalactosyl diglycerides of leaves, whereas Auling et al. (1971) suggested that the positional specificity of fatty acids in the monogalactosyl diglycerides of various plants was related mainly to the chain length rather than to the degree of unsaturation. Furthermore, Safford and Nichols (1970) demonstrated that the positional distribution of fatty acids within the monogalactosyl diglyceride from various algae was influenced more by fatty acid chain length than by their degree of unsaturation, but that in the leaves of most higher plants the positional distribution was based primarily on the degree of unsaturation of the constituent fatty acids. Glycolipids have also been found in numerous bacteria as well as in A. laidlawii B. Here again some conflicting results have recently been reported. The study of Saito and Mukoyama (1971) indicated that, in contrast to the usual situation, longer-chain branched and saturated fatty acids were esterified mainly at the 1-position and shorter-chain branched and saturated acids mainly at the 2-position in diglucosyl diglycerides of Bacillus cereus. Fischer et al. (1973a) reported that in the phosphoglucolipids from Streptococcus hemolyticus, the fatty acids were randomly distributed, but that the compounds from S. faecalis showed positional specificity with the longer-chain fatty acids exhibiting a preference for the 1-position and the shorter-chain fatty acids for the 2-position, regardless of the degree of unsaturation.

In this chapter I will report the results of a systematic investigation of the fatty acid positional specificity in the two major neutral glycolipids (monoglucosyl and diglucosyl diglycerides, MGDG and DGDG, respectively) of *A. laidlawii* B. Essentially the same approach used by McElhaney and Tourtellotte (1970b) was employed in this investigation. The goal of this study was to determine if the fatty acid positional specificity of the neutral glycolipids of *A. laidlawii* B is similar to that previously reported for phosphatidylglycerol of this organism, and to compare the fatty acid positional distribution of the glycolipids of this simple procaryotic organism with that observed in bacterial and plant glycolipids.

The position of the double bond in an unsaturated fatty acid can markedly affect the physical properties of that acid. The incorporation of various positional isomers of a given monounsaturated fatty acid into the membrane lipids would thus produce membrane lipids with a wide range of phase transition temperatures. Therefore, the positional specificity of various positional isomers of *cis*-octadecenoic acid in the phosphatidylglycerol of *A. laidlawii* B were also investigated in order to gain additional insight into the mechanism by which this organism specificially positions fatty acids in this phospholipid.

I have also investigated the effect of varying the amount of a particular fatty acid present in the membrane polar lipid on the positional distribution of that acid, and determined the effect of the incorporation of one exogenous fatty acid on the apparent positional specificity of a second exogenous fatty acid which is being simultaneously incorporated into the membrane polar lipids.

Using the results of the above studies, I will speculate as to the biological significance of the positional specificity of fatty acyl groups in membrane lipids, and I will discuss the mechanisms by which the non-random distribution of fatty acyl groups in A. laidlawii B membrane lipids may be accomplished.

A. The positional distribution of various exogenous fatty acids in the neutral glycolipids

1. Materials and Methods

(a) Culture method: Ethanolic solutions containing various quantities of the fatty acid to be tested (final concentration of 0.1 to 0.6 mM) and appropriate amounts of 16:0 (final concentration of 0.6 to 0.11 mM) were added to 750 ml of lipid-poor growth medium before inoculation. The total concentration of exogenous fatty acids was always 0.12 mM, and the ratio of the exogenous acids were manipulated such that 16:0 always comprised more than 50 mole per cent of the total fatty acids in the membrane lipids. Cells were harvested in late-log or early-stationary phase as described previously.

(b) Purification of the lipids: Total membrane lipids were extracted from the cell pellets, and monoglucosyl- and diglucosyldiglyceride (MGDG and DGDG, respectively) were purified by Unisil column chromatography followed by TLC, using the procedure described in Chapter II of this dissertation, except that different solvent systems were utilized for TLC. The solvent systems used for the purification of MGDG was chloroform-methanol-acetic acid (80:10:4 by vol.), and chloroform-methanol-acetic acid-water (80:13:4:1 by vol.) for the first and second TLC separations, respectively. The solvent system used to purify DGDG by TLC was chloroform-methanol-acetic acid-water (70:20:4:2 by vol.).

(c) Position-specific enzymatic hydrolysis of the glycolipids: MGDG and DGDG were dried overnight in a vacuum chamber and suspended in 0.75 ml of 0.05 M maleate buffer, pH 6.8, containing 4.5 mg of a detergent, Triton X-100. This suspension was then sonicated for 5 minutes in an ice bath using a Biosonik III (Bronwill Scientific, Rochester, N.Y., U.S.A.) equipped with the smallest probe tip, at a setting of 25. To the resulting emulsion were added 0.15 ml of 0.1 M $CaCl_2$ and 0.75 ml of a lipase (EC 3.1.1.3) from Rhizopus arrhizus delemer dissolved in the same buffer but without the detergent. The final concentration of the enzyme was 5 mg/ml and 10 mg/ml for the hydrolysis of MGDG and DGDG, respectively. Immediately after the addition of the enzyme, 0.3 ml of the reaction mixture was withdrawn and the lipids were extracted therefrom by the procedure described below. The reaction mixtures, containing either MGDG or DGDG, were incubated at room temperature or at 37°C for one or two hours, respectively.

(d) Separation of the reaction products: The lipids were extracted from the reaction mixture by a procedure which was essentially the last step of the Bligh-Dyer lipid extraction procedure (1959). Water, chloroform and methanol were added to the reaction mixtures to give a final ratio of 1.7:4.0:2.0, and this solution was centrifuged at 1000 x g for 15 minutes. The lower chloroform layers were taken to dryness, dissolved in a small volume of chloroform-methanol (2:1 by vol.), and applied to a TLC chromatoplate. The solvent systems employed for development were chloroform-methanol-acetic acid-water (80:13:4:1 by vol.) and (70:20:4:2 by vol.) for the MGDG and DGDG, respectively. The locations of the lipids were visualized by exposing the plates to iodine vapor. After decolorizing, the reaction products (free fatty acids and lysoglycolipids) as well as any substrate remaining were extracted from the silica gels by the procedure described previously. Methyl esters of fatty acids were prepared by the conventional procedure and the fatty acid composition of each fraction was analyzed by GLC.

(e) Calculation of the positional specificity: Since the lipase from R. arrhizus delemer hydrolyzes specifically the ester linkage at the 1-position of the glycerol moiety, the fatty acids in the free fatty acid and lysoglycolipid fraction represents the acids esterified at the 1- and the 2-position, respectively, of the neutral glycolipid. The positional specificity of a fatty acid, here expressed as a ratio of the quantity of that fatty acid present at position 2 relative to position 1 in the neutral glycolipid, was calculated by the following equation:

$$\text{Positional specificity } (P_2/P_1) = \frac{\text{mole per cent of the acid in question in lysoglycolipid fraction}}{\text{mole per cent of the acid in question in free fatty acid fraction}}$$

(f) Analytical methods: Phosphorus was determined by the procedure of Chen et al. (1956) using ascorbic acid as the reductant. Fatty acid ester groups were determined by the hydroxylaminolysis procedure of Snyder and Stephens (1959). Carbohydrate contents were analyzed by the anthrone method of Radin et al. (1955).

2. Results

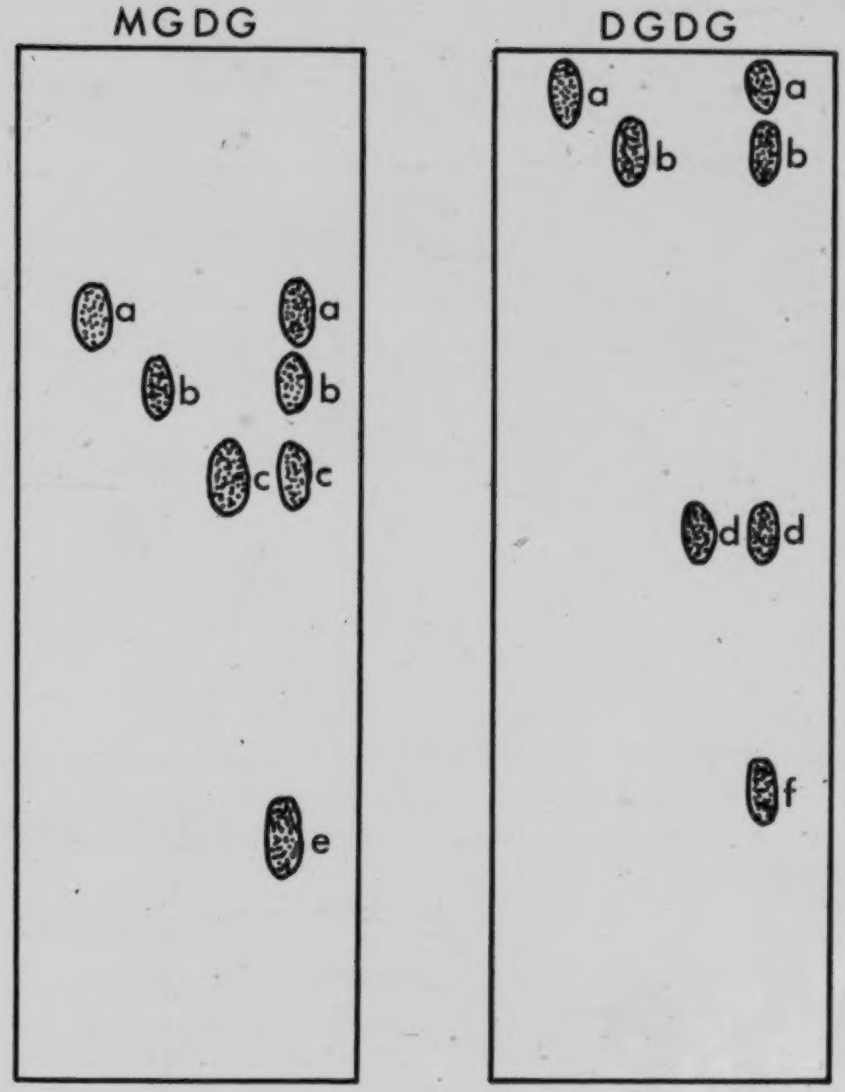
(a) Purity of the glycolipids: MGDG and DGDG fractions were chromatographed on TLC plates with the solvent systems chloroform-methanol-acetic acid-water, 80:13:4:1 and 70:20:4:2, respectively, to check the purity of these glycolipids. Chromatography revealed a single spot in each case with the Rf values of 0.57 and 0.55 for the MGDG and DGDG, respectively. Chemical analysis gave a carbohydrate-fatty acid ester-phosphate molar ratio of 1.00:1.99:0.01 for the MGDG fraction and 1.00:0.94:0.01 for DGDG fractions, consistent with the expected values of 1.00:2.00:0.00 and 1.00:1.00:0.00, respectively.

(b) Confirmation of the positional specificity of the lipase from *Rhizopus arrhizus delemer*: This enzyme showed a very high positional specificity toward the terminal ester linkages of synthetic triglycerides, 1,3-dipalmitolein and 1,2-dipalmitolein, both of which were products of Analabs (North Haven, Conn., U.S.A.). About 97 mole per cent of the free fatty acids and 99 mole per cent of the monoglyceride fatty acids obtained from 1,3-dipalmitolein were 16:0 and 9-18:1c, respectively; about 95 mole per cent of the monoglyceride fatty acids obtained from 1,2-dipalmitolein were 16:0. Furthermore, Fischer et al. (1973b) convincingly demonstrated that this enzyme was also highly specific for the terminal ester linkage in various synthetic galactolipids. In order to reveal any erroneous results which might be caused by the specificity of this enzyme toward any particular fatty acid located at the 1-position, the mole per cent of a fatty acid in question in the unhydrolyzed glycolipids fraction was compared to that in the original sample. In most cases these two figures were similar within experimental error, suggesting that this enzyme does not have high specificity toward particular fatty acids.

(c) Hydrolysis of MGDG and DGDG by the lipase from *R. arrhizus delemer*: The substrates were sonicated before the incubation with the enzyme as described in Materials and Methods. Since Hauser (1971) reported some chemical degradation of egg lecithin by sonication, the chromatographic purity of the glycolipid samples and their fatty acid compositions were checked by TLC and GLC, respectively, before and after sonication, using the methods described previously. There was no deacylation or oxidation of unsaturated fatty acids during the sonication procedure and no detectable hydrolytic degradation of the glycolipids. After the hydrolysis by the enzyme, the reaction products were clearly separated by TLC as described in Materials and Methods, and as shown in Fig. 21. Chemical analysis of the unhydrolyzed glycolipids gave a carbohydrate-fatty acid ester molar ratio of 1.00:1.03 for MGMG (lyso-MGDG) and 2.00:1.04 for DGMG (lyso-DGDG), consistent with the expected values of 1.00:1.00 and 2.00:1.00 respectively.

The fatty acyl groups of 2-acyl lyso-compounds are known to undergo chemical migration to the 1-position under certain conditions,

Fig. 21. Tracings of thin-layer chromatoplates showing the separation of MGDG and DGDG and their enzymatic hydrolysis products. The solvent systems used were chloroform-methanol-acetic acid-water (80:13:4:1 by vol.) and (70:20:4:2 by vol.) for the MGDG and DGDG reaction mixtures, respectively. Identification: a, free fatty acid; b, Triton X-100; c, MGDG; d, DGDG; e, MGMG; f, DGMG. The last column of each chromatoplate shows the separation of the components of the actual reaction mixture, the first three columns in each chromatoplate being the appropriate standards.


MGDG
DGDG
a
b
c
e
a
b
d
f

such as alkaline pH (Albright et al., 1973). If this acyl migration occurs during hydrolysis by the lipase, some of the expected reaction product, 2-acyl lysoglycolipid, would be converted to 1-acyl lysoglycolipid; the 1-acyl lysoglycolipid could in turn be attacked by the lipase, giving rise to additional free fatty acid and reduced levels of lysoglycolipid. Under these conditions the free fatty acid fraction would thus no longer contain only fatty acids derived from the 1-position, and the positional specificity data calculated from a comparative analysis of the fatty acid compositions of the R. arrhizus delemer lipase hydrolysis products would produce erroneous results. The number of fatty acid molecules in the free fatty acid and in the lysoglycolipid fractions should be equal if there is no acyl migration during the hydrolysis by the lipase. To test if indeed any acyl migration did occur during the enzymatic hydrolysis, a known amount of an unnatural fatty acid, 17:0, was added to both the free fatty acid and lysoglycolipid fractions as an internal standard and the amount of fatty acid in each fraction was then calculated from the GLC chromatogram. The total molar quantity of fatty acid was found to be very similar for both the free fatty acid and the lysoglycolipid fractions, indicating that no significant acyl migration occurred during the enzymatic hydrolysis under the experimental conditions described earlier.

Ideally, it would be best to have the enzymatic hydrolysis proceed to completion without significant acyl migration. However, when the hydrolysis was permitted to go to completion by using much longer incubation times than described previously, very serious acyl migration was observed, as revealed by an increased quantity of fatty acid in the free fatty acid fractions (FFA) compared to that in lysoglycolipid fractions (MGMG or DGMG). Under the experimental conditions described in the Materials and Methods section, the reaction went from 70 to 90 per cent completion, and the fatty acid composition of the residual glycolipids were similar to those of the original, untreated glycolipids. An example of the fatty acid compositional data from which the fatty acid positional specificities are calculated is shown in Table 21, in which the fatty acid composition of the products of hydrolysis of DGDG enriched in 14:0 (and 16:0) was determined. From these data the positional specificity of 14:0 (relative to 16:0) can be easily obtained, $^{64.2}/13.6 = 4.7$.

(d) The effect of the amount of fatty acid present in the glycolipid on its positional specificity: Various concentrations (0.01 mM to 0.04 mM) of 14:0 were added to the growth medium, along with appropriate amounts of 16:0, so as to maintain the total concentration of exogenous fatty acid at 0.12 mM. Table 22 gives the positional specificity of 14:0 in each case, as well as the mole per cent of this fatty acid present in the substrate DGDG and in the reaction products of the lipase hydrolysis (unhydrolyzed DGDG, FFA, and DGMG). The positional specificity of 14:0 is reasonably constant regardless of the content of that acid in the DGDG, as long as the content is less than 50 mole per cent. When the effect of altering the quantity of other exogenous fatty acids present in both the MGDG and DGDG fractions was investigated, reasonably constant positional specificity values were also obtained, regardless of the quantity of exogenous fatty acid

Table 21. The fatty acid compositions (mole %) of the untreated DGDG, and of the products of the lipase hydrolysis, from cells grown in 14:0 (0.04 mM) and 16:0 (0.08 mM)

Fatty acid found	Untreated DGDG	Unhydrolyzed DGDG	DGMG	FFA	$\frac{\text{DGMG + FFA}}{2}$
12:0	3.0	1.0	6.8	1.1	3.9
13:0	0.6	0.4	1.2	-	0.6
14:0	36.5	37.8	64.2	13.6	38.9
16:0	59.7	60.8	27.8	80.5	54.2
18:0	0.3	-	-	0.9	1.0
9-18:1c	-	-	-	3.9	2.0

[a] The fatty acid compositional values presented in this table are representative values from five separate experiments.

Table 22. The effect of variations in the content (mole %) of 14:0 in the DGDG from *A. laidlawii* B on the positional specificity of 14:0

The content of 14:0 in:					Positional Specificity of 14:0 ($P2/P1$)
Untreated DGDG	Unhydrolyzed DGDG	DGMG	FFA	$\frac{\text{DGMG + FFA}}{2}$	
17.1	17.6	34.3	6.5	20.4	5.3
28.0	26.6	49.2	9.4	29.3	5.2
32.7	34.1	61.2	13.9	37.6	4.4
36.5	37.8	64.2	13.6	38.9	4.7

present in the glycolipids, provided that the fatty acid in question was not present in sufficient quantities to saturate the preferred position.

(e) The positional specificity of various exogenous fatty acids: In order to insure that the positional distribution value determined for a particular fatty acid is a valid indicator of its relative positional specificity, the concentration of that acid in the glycolipids under study should be low enough to avoid saturation of the preferred position. If, for example, a particular fatty acid is very specific for the 2-position, it would be esterified mainly at the 2-position when present in concentrations of less than 50 mole per cent. However, if the 2-position is saturated with that particular fatty acid, any excess amount of the fatty acid which is incorporated into the glycolipid molecule would be forced into the 1-position, and the positional distribution would no longer reflect the actual positional specificity of the acid. To avoid this situation, the positional distribution of all the fatty acids examined in this dissertation were determined under conditions where the fatty acid under study represents less than 50 mole per cent of the total fatty acids in the glycolipids.

The positional distribution of any fatty acid may be relative, depending upon the nature and concentration of the other fatty acid present in the glycolipid molecule, as will be discussed in more detail in a later section of this chapter. Thus, in order to make valid comparisons between the positional specificities of a wide variety of fatty acids, the total fatty acid composition of the glycolipids must be standardized to some degree. In the experiments described here, this standardization was accomplished by adding 16:0 together with the fatty acid in question to the growth medium in such a way that the concentration of 16:0 always exceeds 50 mole per cent in the total fatty acid of the glycolipids. There were several advantages in using 16:0 for this purpose. Firstly, 16:0 is readily incorporated into the glycolipids, and can easily be maintained at levels of more than 50 mole per cent of the total esterified fatty acid. Secondly, 16:0 is not elongated by A. laidlawii B and in fact inhibits the elongation of the other fatty acids, as was shown in a previous chapter of this dissertation. Thirdly, when 16:0 and any other exogenous acid are added to the growth medium, the biosynthesis of fatty acids is markedly reduced and any effect on the positional distribution of the fatty acid in question due to competition from minor endogenous fatty acids tends to be minimal. An example of the suitability of 16:0 for purposes of fatty acid standardization is seen in Table 21, where 14:0 and 16:0 were added to the growth medium at concentrations of 0.04 and 0.08 mM, respectively. The 14:0 and 16:0 together comprise more than 95 mole per cent of the total fatty acids in the glycolipid DGDG. The positional distribution of 14:0, therefore, would accurately reflect the competition between 14:0 and 16:0 for each position in the glycerol moiety.

Tables 23 and 24 give the positional specificity values of various exogenous fatty acids in MGDG and DGDG, respectively. The numbers in the second column in these tables represent the relative positional affinity of a fatty acid as compared to that of 16:0. The

Table 23. The positional specificity of various exogenous fatty acids in the monoglucosyl diglyceride (MGDG) of *A. laidlawii* B

Fatty acid tested	Positional specificity P2/P1	Concentration (mole %) of exogenous fatty acid	
		Untreated MGDG	$\frac{\text{MGMG + FFA}}{2}$
13:0	12.9[a]	38.4[a]	34.7[a]
14:0	4.7	36.5	38.9
15:0	1.2	34.9	33.4
17:0	0.5	23.8	22.2
18:0	0.5	18.8	19.8
16:01	1.8	36.0	40.6
18:01	1.4	17.2	15.4
13:0ai	14.6	18.6	17.9
15:0ai	32.0	32.1	34.4
17:0ai	2.4	33.3	36.1
9-14:1c	9.9	13.8	14.8
9-14:1t	8.4	27.9	29.1
9-16:1c	5.5	21.9	22.3
9-16:1t	1.8	32.7	34.0
9-18:1c	10.0	21.2	25.7
9-18:1t	5.6	28.5	29.6
9,12-18:2t,t	10.3	23.4	19.8
9,12-18:2c,c	12.7	27.4	28.4
9,12,15-18:3c,c,c	15.1	26.5	23.6
9,10-19:0cp,c	13.7	29.2	33.1
9,10-19:0cp,t	10.2	32.4	36.6

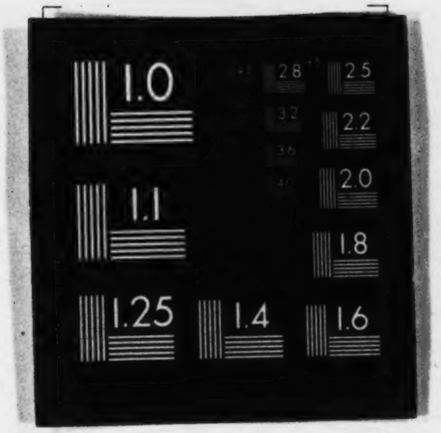
[a] These values are the averages of at least duplicate experiments.

Table 24. The positional specificity of various exogenous fatty acids in the diglucosyl diglyceride (DGDG) of *A. laidlawii* B

Fatty acid tested	Positional specificity P2/P1	Concentration (mole %) of exogenous fatty acid	
		Untreated DGDG	$\frac{DGMG + FFA}{2}$
13:0	10.8[a]	29.2[a]	29.4[a]
14:0	4.9	29.0	31.6
15:0	1.2	39.0	38.4
17:0	0.7	25.1	25.5
18:0	0.7	17.8	19.3
14:0i	5.5	32.9	30.8
16:0i	1.7	44.4	44.7
18:0i	3.1	27.2	29.0
13:0ai	21.1	16.7	16.0
15:0ai	61.0	37.8	37.2
17:0ai	2.5	46.1	50.0
9-14:1c	12.9	14.1	14.5
9-14:1t	8.7	32.7	31.7
9-16:1c	6.4	25.6	25.1
9-16:1t	4.0	38.1	37.0
9-18:1c	12.3	30.0	34.4
9-18:1t	9.1	37.4	41.2
9,12-18:2t,t	13.3	29.0	28.8
9,12-18:2c,c	15.8	39.2	35.6
9,12,15-18:3c,c,c	22.3	31.8	30.9
9,10-19:0cp,c	15.7	37.1	39.5
9,10-19:0cp,t	10.4	36.9	38.7

[a] These values are the averages of at least duplicate experiments.

3 OF/DE 3


1.0
2.8
2.5
3.2
2.2
3.6
2.0
4.0
1.1
1.8
1.25
1.4
1.6

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greater this number, the more specific is a fatty acid for the 2-position. The third column gives the concentration of any particular fatty acid in the untreated glycolipids, as measured experimentally. The fourth column again gives the expected concentration of a particular fatty acid, as calculated by averaging the fatty acid composition values obtained from the lipase hydrolysis products. These two columns are theoretically identical, and the values in the last column serve as an internal control.

In general, any particular fatty acid exhibits a similar positional specificity in MGDG and DGDG. In the series of straight-chain saturated fatty acids, the shorter the chain length, the greater is the specificity for the 2-position. The introduction of a single methylene unit significantly reduces the $^{P2}/P1$ value. In the series of isobranched acids, the affinity for the 2-position is always higher than for the corresponding straight-chain saturated fatty acid. Within this group the shorter the chain length, the greater is the specificity toward the 2-position, with the exception of 18:0i in the DGDG fraction. The anteisobranched fatty acids show a higher specificity toward the 2-position than the corresponding isobranched acids. 15:0ai has an unusually high specificity to the 2-position, for reasons which will be discussed later. In fact, this acid shows the highest specificity toward the 2-position among all the fatty acids tested. The introduction of one or more double bonds markedly increases fatty acid specificity for the 2-position. The _cis_-double bond is more effective as a 2-position director than is the _trans_-double bond. In both the _cis_- and _trans_-monounsaturated series, fatty acids with eighteen carbon atoms have unexpectedly high $^{P2}/P1$ values, the $^{P2}/P1$ ratios being almost the same or sometimes even a little higher than those observed with the monounsaturated fatty acids having fourteen carbon atoms. This will also be discussed later. The introduction of additional double bonds increases the affinity for the 2-position to an even greater extent. The order of specificity is as follows: 9,12,15-18:3c,c,c > 9,12-18:2c,c > 9,12-18:2t,t. The introduction of a cyclopropane ring into a straight-chain saturated fatty acid has a very marked effect on the positional specificity. As was noticed previously with the PG of A. laidlawii B (McElhaney and Tourtellotte, 1970b), the introduction of a cyclopropane ring has a more pronounced effect in increasing the affinity for the 2-position than does the introduction of a *cis*-double bond. In this regard it is of interest to note the reduced total incorporation of exogenous cyclopropane fatty acids as compared to the corresponding monounsaturated acids, which was discussed earlier in this dissertation. In fact, the introduction of a cyclopropane ring has an effect similar to that of the introduction of two double bonds, on the basis of the positional specificity values determined here. Thus 9,10-19:0cp,c and 9,12-18:2c,c have very similar positional specificities, as have 9,10-19:0cp,t and 9,12-18:2t,t.

3. Discussion

The fatty acids tested which have very high positional specificities to the 2-position are the short, straight-chain saturated, short- and medium-chain anteisobranched, monounsaturated, polyunsaturated, and cyclopropane-ring containing fatty acids. Medium chain-length straight-chain and long-chain iso- and anteiso-branched fatty acids have a moderate specificity toward the 2-position. 17:0 and 18:0 are the only fatty acids tested which can successfully compete with 16:0 for the 1-position. The effect of fatty acid structure on positional affinity is more clearly seen if one compares a series of fatty acids containing the same number of carbon atoms but belonging to different chemical classes. The following is the order of fatty acids arranged according to their affinities for the 2-position:

9,12,15-18:3c,c,c > 9,12-18:2c,c , 9,10-19:0cp,c >
9,12-18:2t,t > 9,10-19:0cp,t , 9-18:1c > 9-18:1t >
18:01 > 18:0

Interestingly enough, the same or a very similar order would be obtained if these fatty acids were arranged according to their melting points, with the affinity for position 2 decreasing as the melting point of the fatty acid increases.

These observations lead to the conclusion that the weaker the apolar attractive interactions characteristic of a fatty acid, the greater is its tendency to be esterified at the 2-position. There are some apparent exceptions to this rule: 1. 15:0ai has an exceptionally high specificity to the 2-position; and 2. monounsaturated fatty acids with fourteen and sixteen carbon atoms have a little lower specificity to the 2-position compared to the monounsaturates with eithteen carbon atoms. However, these exceptions might be more apparent than real. The positional specificity value of 13:0ai reported here may not represent the real positional affinity of this fatty acid, which might be even higher than that of 15:0ai. A large portion of the 13:0ai incorporated into the cells is elongated to 15:0ai, as is shown in Table 25. Also, the content of 14:0 and 9-18:1c in the glycolipid is relatively high under these conditions. These factors might also tend to reduce the actual positional specificity value of 13:0ai in these experiments, since 13:0ai would have to compete for the 2-position not only with 16:0 but also with other fatty acids which have appreciable affinities for the 2-position. The positional specificities of 9-14:1c, 9-14:1t and 9-16:1c reported here may also be lower than the actual values, because all of these acids are quite extensively elongated to their respective higher homologues, even in the presence of large amounts of exogenous 16:0. Examples are shown in Table 25. The differences in the relative position of the double bond among these monounsaturated fatty acids with different chain lengths might also make it difficult to make a meaningful comparison of the relative positional specificity values obtained.

Although there are some differences in the content of the various exogenous fatty acids tested in the glycolipids, it is still valid to make a comparison between the positional specificity values of the various fatty acids derived from these experiments. This is true because the positional specificity value of a fatty acid was shown to be constant regardless of its content in the glycolipid, as long as it was present in less than positionally-saturating amounts, as illustrated in Table 22.

The metabolic pathways for the biosynthesis of the glycolipids and phospholipids by A. laidlawii B were discussed previously

Table 25. The fatty acid compositions (mole %) of diglucosyl diglyceride (DGDG) from A. laidlawii B cells grown in the presence of 16:0 plus one of the other exogenous fatty acids listed below

	Exogenous fatty acid other than 16:0			
Fatty acid found	13:0ai	9-14:1c	9-14:1t	9-16:1c
12:0	-	2.4	-	0.8
13:0ai	14.0	-	-	-
14:0	6.8	2.8	3.5	4.6
9-14:1c	-	14.8	-	-
9-14:1t	-	-	33.8	-
15:0ai	6.3	-	-	-
16:0	64.9	59.0	54.1	54.5
9-16:1c	-	-	-	23.3
11-16:1c	-	21.0	-	-
11-16:1t	-	-	8.7	-
18:0	2.9	-	-	0.5
9-18:1c	5.1	-	-	-
11-18:1c	-	-	-	16.3

The fatty acid compositional values presented in this table are representative values from at least duplicate experiments.

in this dissertation (see Fig. 19). Presumably phosphatidic acid is the common precursor for both the glycolipids and phospholipids. All of the polar lipids in this organism are metabolically stable (McElhaney and Tourtellotte, 1970a); there is no deacylation nor reacylation of the polar lipids. Since the positional specificities of the various fatty acids in the glycolipids examined here are quite similar to the positional specificity values of these fatty acids in the major phospholipid (McElhaney and Tourtellotte, 1970b), and since no rearrangement of the fatty acids can occur after their esterification into the membrane polar lipids, the enzymes which convert alpha-glycerophosphate to phosphatidic acid must be solely responsible for the asymmetric positional distribution of fatty acids observed in this organism. Romijn et al. (1972) have recently confirmed that the characteristic asymmetric distribution of exogenous fatty acids is already observed in the phosphatidic acid fraction. The enzyme (or enzymes) which catalyzes the positionally specific esterification of alpha-glycerophosphate apparently functions by recognizing the physical nature of the fatty acid substrate and not by responding to their chemical properties, since chemically different fatty acids with similar melting points have similar positional specificities. For example, the short-chain saturated, short- and medium-chain anteisobranched, cis-monounsaturated, polyunsaturated, and cyclopropane ring-containing fatty acids tested here belong to different chemical groups, yet all of these fatty acids produce phospho- and glyco-lipids with relatively low gel to liquid-crystalline phase transition temperatures, and all have high specificities to the 2-position. Furthermore, within a given group of chemically-related fatty acids, the shorter the chain length of the fatty acid the greater is its affinity for the 2-position.

Most other organisms have a more complicated fatty acid metabolism than does _A_. _laidlawii_ B, and the biochemical reactions which are responsible for the final asymmetric fatty acid positional distribution are rather ambiguous. For example, in _Chlorella_ _vulgaris_ the apparent positional distribution of the fatty acids within the monogalactosyl diglyceride was influenced more by fatty acid chain length than by the degree of unsaturation, with appreciable amounts of unsaturated C18 acids accumulating preferentially in the 1-position and C16 acids in the 2-position (Safford and Nichols, 1970). However, it was suggested by these investigators that some desaturation of fatty acids occurred at the level of the intact glycolipid, and that the original glycolipids contained predominantly 18:0 and 16:0 at the 1- and the 2-positions, respectively. If this suggestion is correct, then the "original" positional distribution of fatty acids in this glycolipid is rather similar to that of other organisms, despite the apparent difference in the distribution of fatty acids in the final glycolipid product. This complicated situation does not occur in _A_. _laidlawii_ B. Cyclopropane fatty acids are generally found predominantly at the 2-position (Van Deenen, 1966). However, this again does not reflect a positionally specific esterification of the cyclopropane fatty acids, since cyclopropane acids are synthesized from monounsaturated fatty acids at the level of the intact polar lipids (Thomas and Law, 1966). The high specificity of cyclopropane acids for the 2-position is then due to the positional specificity of the monounsaturated fatty acid precursors, unless a significant subsequent redistribution of fatty acids were to occur. In contrast, the apparent distribution of cyclopropane acids in A. laidlawii B simply reflects the positionally specific esterification of these fatty acids, because this organism does not synthesize cyclopropane acids.

It is of interest to note that there are at least two exceptions reported to the general rule for fatty acid positional distribution in phospholipids: phosphatidylethanolamine of Clostridium butyricum and phospholipids of Mycobacteria. In C. butyricum unsaturated and cyclopropane fatty acids were found in more abundance at the 1-position (Hildbrand and Law, 1964). Since this organism synthesizes unsaturated fatty acids anaerobically via beta-hydroxydecanoyl-ACP and not by the direct oxidative desaturation at the level of intact phospholipids, this apparent positional distribution should truly reflect the positional specificity of the fatty acid esterification step. In cardiolipin and phosphatidylethanolamine of several Mycobacteria, 16:0 was mainly located at the 2-position, while 9-18:1c and 10-methyl-18:0 were esterified at the 1-position (Okuyama et al., 1967). However, since these organisms can produce unsaturated fatty acids by direct oxidative desaturation, they may be able to desaturate fatty acids at the level of intact phospholipids, as does Chlorella vulgaris (Gurr et al., 1969). If this is correct, the apparent fatty acid positional specificity observed in certain Mycobacteria does not necessarily reflect the positional specificity of the initial esterification of alpha-glycerophosphate, the actual specificity of which might be rather similar to that observed in other organisms. It is also not surprising that 10-methyl-18:0 showed the same positional distribution as 9-18:1c, because the former is synthesized from the latter by methylation with S-adenosyl methionine at the level of the intact phospholipid (Akamatsu and Law, 1970).

Whatever the enzymatic reactions responsible for the fatty acid positional specificity, the fact remains that a markedly non-random distribution of fatty acids has been observed in nearly every polar lipid which has been studied to date, and in most cases the relative positional affinities of the component fatty acyl groups seem to be determined by their physical properties, in particular by their relative melting points. With this knowledge of the physicochemical principle underlying this phenomenon, we can now speculate as to its biological significance. Phillips et al. (1972) have studied the inter- and intra-molecular mixing of hydrocarbon chains in lecithin-water systems by differential scanning calorimetry. These investigators found that when pure 1,2-diacyl lecithins containing a different type of hydrocarbon chain in each position of the molecule (intra-molecular chain mixing) are dispersed in excess water, a single gel to liquid-crystalline phase transition is observed which lies between the two transition temperatures expected from the two pure lecithins containing exclusively either one of the two fatty acyl chains in the mixed-chain molecule. When two different lecithins, each containing only one type of hydrocarbon chain, are mixed (inter-molecular mixing), two phase transitions are observed. The phase transition of the higher-melting lecithin becomes broader and occurs at a lower temperature than is observed if the higher-melting phospholipid were present alone, although the transition temperature of the lower-melting was not affected by the presence of the higher-melting lecithin. If fatty acids of rather dissimilar melting points were to be randomly positioned in membrane lipids, half of the total lipid would be made up of molecular species which would contain two higher-melting or two lower-melting fatty acyl groups, and the other half of the membrane lipids would contain both a higher- and a lower-melting fatty acid. In this case, co-crystallization of these three molecular species would not occur at physiological temperatures and appreciable quantities of the membrane lipid would exist in the gel state. Evidence exists that the excessive local crystallization of lipids in biological membranes can disrupt normal cellular growth as well as membrane-associated enzymatic and transport functions. On the other hand, the existence of enzyme systems, which synthesize almost exclusively polar lipids containing two fatty acids having rather different melting points, results in the production of a more physicochemically homogeneous mixture of molecular species which are largely comiscible within the physiological temperature range.

B. The effect of the position of the double bond in a monounsaturated fatty acid on its positional distribution in a phospholipid

1. Materials and Methods

(a) Culture method: This was described in the first section of this chapter.

(b) Purification of phosphatidyl glycerol (PG): Total membrane lipids were extracted from the cell pellets, and PG was purified by Unisil column chromatography followed by TLC by the procedure described in Chapter II of this dissertation, except that the solvent system employed for the TLC was chloroform-methanol-conc. NH_4OH (65:30:5).

(c) Hydrolysis of PG by a lipase: PG was dried overnight in a vacuum chamber, suspended in 1 ml of 0.05 M maleate buffer, pH 6.8, containing 6 mg of a detergent, Triton X-100, and sonicated for 30 seconds in an ice bath as described previously. To the resulting emulsion were added 0.2 ml of 0.1 M $CaCl_2$ and 1 ml of lipase from _R_. _arrhizus_ _delemer_ dissolved in the same buffer without detergent. The final concentration of the enzyme was 1 mg/ml. Slotboom _et_ _al_. (1970) showed that this enzyme is also able to hydrolyze the fatty acid ester bond exclusively at the 1-position of a number of synthetic phosphoglycerides, including PG, regardless of the nature and distribution of the fatty acid constituents. Immediately after the addition of the enzyme, 1 ml of the reaction mixture was removed and placed in 10 ml of isopropanol to stop the enzyme reaction (Fischer _et_ _al_., 1973b). The rest of the mixture was incubated at 37°C for 1 hour with vigorous shaking, and then transferred to 10 ml of isopropanol. Known amounts of 17:0 were added to both the zero time and 1 hour samples as an internal standard, and both samples were evaporated to dryness. Isopropanol not only stops the enzyme reaction, but also facilitates the evaporation of water from the reaction mixture by flash evaporation. The routine Bligh-Dyer method could not be successfully applied here to extract lipids from the reaction mixture because the lyso-PG (LPG) fraction did not partition well into the chloroform layer.

(d) Separation of the reaction products: The dried reaction products (both zero time and 1 hour samples) were dissolved in 0.4 ml of chloroform-methanol (2:1) and separated by TLC using the solvent system chloroform-methanol-acetic acid-water (65:25:4:2 by vol.). The locations of the lipids were visualized by exposing the plates to iodine vapor. After decolorizing, the reaction products (FFA, LPG, and PG at zero time) were extracted from the gels by the procedure described previously. From the TLC plates on which zero time samples were separated, areas of the silica gel corresponding to potential locations of FFA and LPG were also scraped off and extracted with methanol, in order to detect the presence of any lipoidal background materials which might give erroneous results. A known amount of 17:0 was added to the LPG fractions of both zero time and 1 hour samples as an internal standard. Methyl esters of fatty acids were prepared from each fraction by the method described previously, and the fatty acid compositions were analyzed by GLC.

(e) Calculation of the positional specificity: The small amounts of fatty acid detected in the zero time FFA and LPG fractions, which are apparently derived from impurities in the silica gel, were subtracted from the corresponding fractions of the 1 hour sample to arrive at a corrected fatty acid composition for these fractions. The positional specificity values were then calculated as described in a preceding section of this chapter.

(f) Analytical methods: The purity of the octadecenoate isomers was checked by the following procedures: (1) methyl esters of each sample were checked on TLC using the solvent system hexane-diethyl ether-acetic acid (90:10:1 drop by vol.); (2) methyl esters were also checked by GLC; (3) the positions of double bonds were analyzed by the Von Rudloff oxidation method (Jones and Davison, 1965) using $NaIO_4$ and $KMnO_4$ as the oxidants, followed by a GLC analysis of the resultant mono- and di-carboxylic acids. All the isomers used except 17-18:1c were at least 90 per cent pure. The 17-18:1c was about 80 per cent pure, and its major contaminant was 16-18:1c.

2. Results

(a) Purity of PG samples: The sample PG was routinely checked by TLC, using the solvent system chloroform-methanol-acetic acid-water (65:25:4:2 by vol.) to check its purity. The samples' consistently produced the Rf value of 0.50. The commercial PG standard (Serdary Research Laboratories, London, Ont., Canada) gave the same Rf value. The chemical analysis gave a fatty acid ester-phosphate molar ratio of 2.05:1.00, very close to the expected value of 2.00:1.00.

(b) Hydrolysis of PG by the lipase from R. arrhizus delemer: The substrates were sonicated before the incubation with the enzyme as described in Materials and Methods. There was no deacylation nor oxidation of unsaturated fatty acids during the sonication, as checked by TLC and GLC, respectively.

After hydrolysis by the enzyme, the reaction products were cleanly separated on TLC as described in Materials and Methods. The Rf values for FFA, PG, and LPG were 0.96, 0.50 and 0.22, respectively. The hydrolysis went to completion, and there was no significant acyl migration under the experimental conditions described, as confirmed by the very similar total fatty acid mole fraction values of the FFA and LPG fractions, after correction to the internal standard.

(c) The positional specificities of various octadecenoate isomers in PG: The distribution of the various positional isomers of octadecenoic acid between the 1- and 2-positions of PG are presented in Table 26. The mole per cent of the octadecenoic acid under study in the substrate PG, and the average between the two products (LPG and FFA fractions) are also included in the second and third columns in this table, respectively. The agreement between these two columns is generally good, suggesting that no significant acyl migration occurred during the enzymatic hydrolysis. As a general rule, the affinity of an octadecenoate isomer for the 2-position increases markedly as the position of the double bond comes closer to the carboxyl end of the molecule, although there are several exceptions. The positional specificity values of the series of positional isomers from 7-18:1c to 17-18:1c are plotted against the position of the double bonds in Fig. 22. A very distinctive effect of double bond location on the positional specificity is clearly demonstrated here. The positional specificity values decrease linearly from 7-18:1c to 17-18:1c. 7-18:1c is very specific for the 2-position, with about 95 per cent of this isomer being esterified at position 2, whereas 17-18:1c has a higher affinity for the 1-position, only 33 per cent of this isomer being esterified at the 2-position.

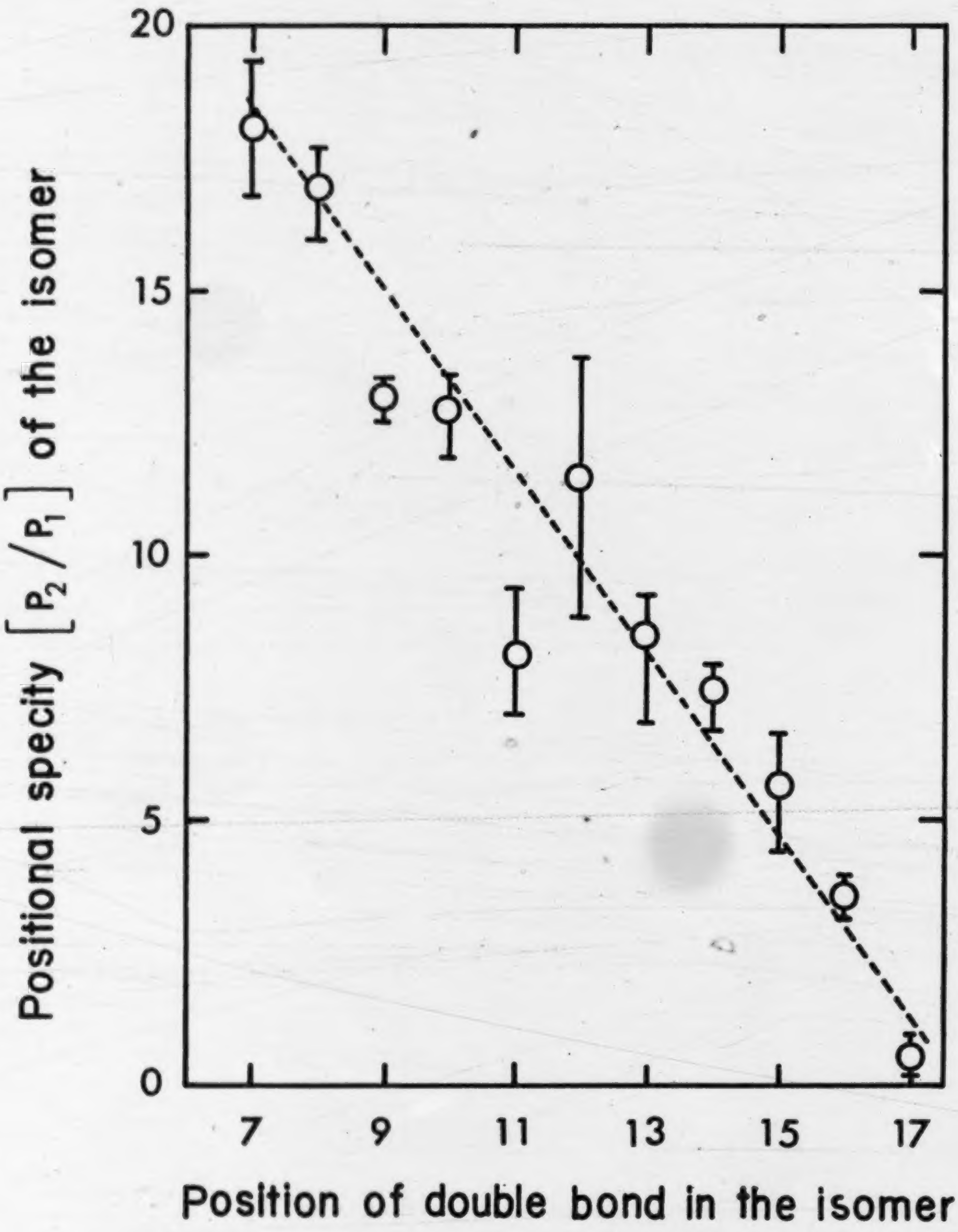
Table 26. The positional specificities ($^{P2}/P1$ values) of various octadecenoate positional isomers in phosphatidylglycerol from A. laidlawii B

Position of double bond	Concentration of acid in question in: PG	Concentration of acid in question in: $\frac{LPG + FFA}{2}$	Positional Specificity $^{P2}/P1$
3	40.7[a]	38.8	∞[b]
4	38.6	39.0	∞
5	35.4	31.3	4.9
6	36.9	35.0	6.4
7	38.6	40.1	18.1
8	31.0	34.1	17.0
9	28.1	28.5	13.0
10	31.7	27.8	12.7
11	30.3	32.2	8.1
12	31.8	35.3	11.5
13	35.1	39.2	8.5
14	33.5	36.2	7.5
15	35.8	38.4	5.7
16	33.6	36.6	3.6
17	20.1	20.2	0.48

[a] The concentration of fatty acid is expressed in mole % and all values are the averages of triplicate experiments.

[b] more than 20

Fig. 22. The effect of the position of the double bond in cis-octadecenoic acid on the positional specificity of that isomer in PG. A. laidlawii B cells were grown in the presence of one of the various positional isomers of cis-octadecenoic acid plus 16:0 as the standard competing fatty acid. PG was extracted from the cell membrane and the positional specificity value (P2/P1 ratio) of each isomer in the PG was calculated as described in the text. The values presented are averages of triplicate experiments. The variation in the results obtained for each isomer are indicated by vertical bars.

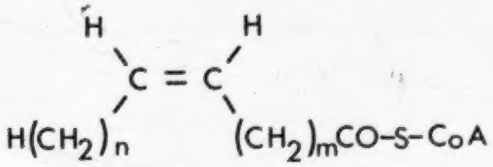

20
15
10
5
0
7
9
11
13
15
17
Positional specity $\left[P_2/P_1\right]$ of the isomer
Position of double bond in the isomer

3. Discussion

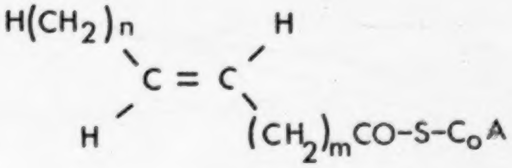
McElhaney and Tourtellotte (1970b) have studied the positional specificity of a variety of fatty acids in the PG of *A. laidlawii* B using *Ophiophagus hannah* venom as the source of phospholipase A. They found an apparent correlation between the relative strength of the apolar interaction of a particular fatty acid and its positional specificity: the stronger the apolar attractive forces, the more specific was a fatty acid for the 1-position. From this conclusion it would be expected that the position of the double bond in the fatty acid molecule would also affect its positional specificity, because the position of the double bond has some effect on the physical properties of fatty acids. The relative strengths of the apolar interactions of the various octadecenoate isomers, when incorporated into membrane lipids, might be approximated by their capillary melting points. Thus the lower the melting point of the free acid, the weaker would be the apolar interactions of the phospholipid molecules when these fatty acids are incorporated into membrane lipids. The fatty acid having a *cis*-double bond at the center of the molecule is known to have the lowest capillary melting point among the octadecenoic acid positional isomers (Gunstone, 1967). The gradual decrease of the positional specificity values from 9-18:1c to 17-18:1c correlates with the increase of the capillary melting points, and confirms the generalized rule proposed by McElhaney and Tourtellotte (1970b). The gradual decrease of the positional specificity values, however, does not correlate precisely with the increase of the melting points, which were found to alternate between the odd-numbered and the even-numbered positional isomers. These alterations in melting points were explained by the differential tilts of the hydrocarbon chain with respect to the plane of the terminal methyl group in the crystalline phase. Incidentally, the decrease in the positional specificity with increasing chain lengthening of the straight-chain saturated fatty acids noted in the first section of this chapter also does not follow the alternating capillary melting points between odd- and even-numbered fatty acids. The capillary melting points, which reflect the hydrocarbon chain interactions in the solid phase, probably are not directly relevant to the physical situation which occurs during polar lipid biosynthesis in the plasma membrane of A. laidlawii B. The positional specificity values of the octadecenoate isomers from 3-18:1c to 8-18:1c do not correlate with the capillary melting points of these isomers at all. For example, 3-18:1c is expected to have almost the same positional specificity as 17-18:1c according to its capillary melting point. However, the former is almost absolutely specific for the 2-position, and the latter is rather more specific for the 1-position. I feel that the capillary melting points of the octadecenoic isomers, which have the double bond located in the carboxyl half of the molecule, may no longer necessarily reflect the strength of apolar interactions of these acids when incorporated into the membrane lipids under the experimental conditions. In a fully hydrated lipid bilayer structure, a cis-double bond in a fatty acyl chain is expected to give a "kink" in the fatty acyl chain, which can no longer be as easily accommodated as in the anhydrous, free fatty acid crystal. The closer the "kink" to the carboxyl end of the fatty acid molecule, the more strongly might it inhibit the apolar interactions of the lipid molecules in the bilayer structure. If this reasoning is correct, then 3-18:1c would be expected to have the highest specificity for the 2-position, and the results obtained here would again confirm the general rule proposed by McElhaney and Tourtellotte (1970b). Of course the validity of the hypothesis should be tested by checking the fluidity of chemically synthesized lipids containing 16:0 and the various positional isomers of *cis*-octadecenoic acid, but preliminary experiments seem to confirm that in mixed-chain phospholipids, the *cis*-double bond is most disruptive when located nearest the carboxyl group (P.G. Barton, personal communication). The abrupt change of positional specificity values between 4-18:1c and 5-18:1c is, incidentally, qualitatively correlated with a region of abrupt change in the capillary melting points. However, the reason for the apparently anomalous behavior of the above isomers is not understood at present.

The only monounsaturated fatty acid overlapping between the present series of experiments and those of McElhaney and Tourtellotte (1970b) is 9-18:1c. The agreement between the positional specificity values of this fatty acid obtained by the two different procedures is very good.

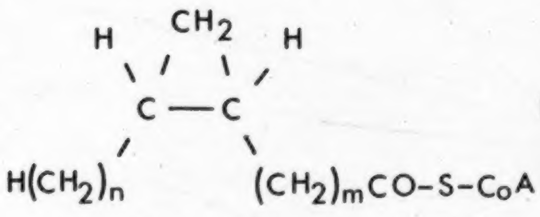
Unlike *A. laidlawii* B, most higher organisms as well as *E. coli* can deacylate and reacylate intact phospholipids. Lands and Hart (1965) showed that the liver microsomal acyl-Coenzyme A:phospholipid acyltransferase has a very distinctive fatty acyl-CoA specificity, which depends upon whether the acceptor used is a 1-acyl phospholipid or a 2-acyl phospholipid. When the former was the acceptor, 9-18:1c-CoA was the best substrate; when the latter was the acceptor, 18:0-CoA was the most suitable. These results correspond to the fatty acid positional distribution observed in the natural phospholipids of the liver, and suggest that this enzyme or enzymes might be responsible for the positional distribution of fatty acids in phospholipids, at least in part. To gain a deeper understanding of this enzyme, a series of experiments have been performed using the chemically synthesized positional isomers of *cis*-octadecenoyl-CoA, *trans*-octadecenoyl-CoA, *cis*-methylene-octadecanoyl-CoA, and octadecynoyl-CoA (Reitz *et al.*, 1969; Okuyama *et al.*, 1969; Okuyama *et al.*, 1972; Tamai *et al.*, 1973). Their chemical structures are illustrated below:



cis-octadecenoyl-CoA



trans-octadecenoyl-CoA



cis-methylene-octadecanoyl-CoA

$H(CH_2)_nC \equiv C(CH_2)_mCO\text{-}S\text{-}CoA$

octadecynoyl-CoA

The comparisons of Vmax values were made among isomers using both 1-acyl and/or 2-acyl phospholipids as the acceptors. It was clearly demonstrated that the enzyme or enzymes could distinguish between the positional isomers within a series, and that they exhibit very different Vmax values for the different isomers. However, none of the results showed a gradual linear decrease or increase of Vmax values when the functional group was moved from the carboxyl to the methyl end of the fatty acid molecule. For example, acyl-CoA:1-acyl-glycerophosphorylcholine acyltransferase and acyl-CoA:2-acyl-glycerophosphorylcholine acyltransferase activities showed a distinct preference for the 9- and 12-isomers, respectively, when a series of _cis_-octadecenoyl-CoA's were examined (Reitz _et al_., 1969). It was also noted that both activities were extremely low for acyl-CoA's having a _cis_-double bond closer than 7 carbon atoms from the carboxyl end. The ratio of the Vmax values between these two activities for each isomer was obtained and compared as an index for the positional specificity. Here again no apparent gradual linear decrease or increase was observed when the functional group was moved along the molecule from the carboxyl to the methyl end. Of course, as Okuyama and Lands (1972) have demonstrated, these specificities might be changed if different concentrations of the monoacyl phospholipids are used, and they might not represent the situation _in vivo_. However, the selective recognition of fatty acids having double bonds at the 9 and 12 positions could certainly be relevant for higher organisms, which contain appreciable amounts of polyunsaturated fatty acids with the double bonds located at these positions.

C. The relative nature of fatty acid positional specificities

1. Materials and Methods

The experimental approach used here is essentially the same as that discussed in the first section of this chapter, except that other exogenous fatty acids replace 16:0 as the standard fatty acid against which the other exogenous fatty acids compete. In the first series of experiments, 16:0 was replaced by 9-18:1t and the positional distribution of various exogenous fatty acids in diglucosyl diglyceride (DGDG) was examined. In the second series of experiments, 16:0 was replaced by various different fatty acids, and the positional distribution of 14:0 in DGDG was studied.

2. Results

In the first series of experiments the positional specificity of several fatty acids in DGDG was determined under conditions where 9-18:1t comprised more than 50 mole per cent of the total esterified fatty acid. The results of these experiments are presented in Table 27. This table also includes the positional specificity values of these acids when 16:0 is the major fatty acid component, and the "theoretical" positional specificity values, which will be discussed later. The positional specificity values, defined as the $^{P2}/P1$ ratios, of all the acids tested are drastically reduced by replacing 16:0 with 9-18:1t as the major fatty acid constituent. That is, the relative affinities of all exogenous fatty acids tested for the 1-position are increased when a lower melting fatty acid, 9-18:1t, is present and presumably competing

Table 27. The positional specificities (^{P2}/P1 values) of various exogenous fatty acids in the diglucosyl diglyceride (DGDG) from A. laidlawii B when 9-18:1t comprises more than 50 mole per cent of the total esterified fatty acid

Fatty acids tested	Concentration (mole %) of exogenous fatty acid		^{P2}/P1 values		
	DGDG	$\frac{\text{DGMG + FFA}}{2}$	found	16:0[a]	theoretical[b]
13:0	6.2[c]	5.3	1.7	10.8	1.2
14:0	17.8	18.2	0.6	4.9	0.5
15:0	26.0	30.8	0.1	1.2	0.1
15:0ai	25.0	22.0	15.6	61.0	6.7
17:0	31.9	35.5	0.9	2.5	0.3

[a] The positional specificity values determined experimentally when 16:0 comprises more than 50 mole per cent of the total fatty acid

[b] The positional specificity values of each fatty acid in DGDG expected theoretically when 9-18:1t replaces 16:0 as the exogenous fatty acid standard calculated as described in the text

[c] All values presented in this table are the averages of triplicate experiments.

more effectively for the 2-position. It is of interest to note that 14:0, 15:0 and 17:0ai, which are predominantly located at the 2-position when large amounts of 16:0 are simultaneously incorporated into the glycolipids, are now found primarily at the 1-position when 9-18:1t is present in the growth medium. In the second series of experiments several fatty acids other than 16:0 or 9-18:1t were used as the major fatty acid constituent, and the positional specificity of 14:0 in DGDG was studied in each case. The results of these experiments are shown in Table 28. This table also includes the "theoretical" $^{P2}/P1$ values, as will be discussed later. It can be clearly seen that the positional specificity of 14:0 can vary markedly in response to the physico-chemical properties of the other exogenous fatty acid present in the growth medium. The higher the melting point of the major exogenous fatty acid, the more effectively it competes with 14:0 for esterification at position 1 and the higher the resultant $^{P2}/P1$ value of 14:0.

3. Discussion

The positional specificity values obtained in the first section of this chapter should represent the apparent positional affinities of a variety of exogenous fatty acids relative to 16:0. Since the $^{P2}/P1$ values obtained are relative ones, I would predict that the apparent positional affinities of any exogenous fatty acid should be shifted in the appropriate direction by altering the nature of the second exogenous fatty acid used as a standard. This is of course just what is observed experimentally. We can go further and

Table 28. The positional specificity ($^{P2}/P1$ value) of 14:0 in diglucosyl diglyceride (DGDG) when various other exogenous fatty acids comprise more than 50 mole per cent of the total esterified fatty acid

Fatty acids tested	Concentration (mole %) of 14:0		$^{P2}/P1$ ratio	
	DGDG	$\frac{\text{DGMG + FFA}}{2}$	found	theoretical[a]
15:0	25.9[b]	26.4	2.4	4.0
16:0	29.0	31.6	4.9	-
17:0	31.6	30.8	7.0	6.8
15:0ai	23.7	24.2	0.2	0.1
17:0ai	22.9	25.3	0.9	1.9
9-18:1t	17.8	18.2	0.6	0.5

[a] The positional specificity values ($^{P2}/P1$) of 14:0 in DGDG expected theoretically when various fatty acids other than 16:0 comprise more than 50 mole per cent of the total esterified fatty acid calculated as described in the text

[b] All values presented in this table are the averages of duplicate experiments.

predict the approximate "theoretical" $^{P2}/P1$ value for any combination of two exogenous fatty acids from our knowledge of the $^{P2}/P1$ value of those acids relative to 16:0. Since, for example, 9-18:1t is roughly nine times more specific for the 2-position than 16:0, we can arrive at a simple-minded prediction of the $^{P2}/P1$ value for any acid relative to 9-18:1t, simply dividing the $^{P2}/P1$ value obtained in the 16:0-standardized experiments by nine. This was in fact done to generate the "theoretical" $^{P2}/P1$ values presented in Table 27. The agreement between the actual and "theoretical" positional specificity value is generally good, especially considering that some error due to a generally altered total fatty acid composition would be expected to influence the experimental values. The agreement between the calculated and observed values supports the concept that the relative positional affinity of any given fatty acid is determined primarily by a simple competition with other fatty acids undergoing simultaneous incorporation into the membrane lipid.

In the second series of experiments, several other fatty acids were used instead of 16:0 or 9-18:1t as the major exogenous fatty acid constituent, and the influence of these fatty acids on the positional specificity of a single fatty acid, 14:0, was determined. The "theoretical" positional specificity values of 14:0 in each experiment were again predicted by simply dividing the positional specificity value of 14:0 relative to 16:0 by that of each other major exogenous fatty acid, again relative to 16:0. The agreement between the experimental and the predicted values is generally good, although again there are some discrepancies noted, which are probably due to differences in the total fatty acid compositions. The positional specificity of 14:0 changes drastically depending upon the physicochemical nature of the major exogenous fatty acid. When 17:0 is the major fatty acid about 88 per cent of the 14:0 is found at the 2-position, but when 15:0ai is the major exogenous fatty acid only 14 per cent of the 14:0 is found at the 2-position.

The results of these two series of experiments strongly support the conclusions reached in the experiments performed with 16:0 as the major exogenous fatty acid. They demonstrate further that the positional specificity of any particular fatty acid is not fixed by its chemical or physical nature but rather is flexible, depending upon the over-all fatty acid composition of the membrane lipids. Although these experiments were done only with DGDG and a limited number of fatty acids were tested, these results are very probably general, at least for the other membrane lipids of this organism. The enzyme system which catalyzes the acylation of sn-glycerol-3-phosphate to phosphatidic acid is both highly specific with regard to placement of fatty acyl groups and yet this specificity is manifested in a very flexible manner. Thus this enzyme system can differentiate between saturated fatty acids differing by only a single methylene group and yet is capable of catalyzing the esterification of fatty acids of intermediate melting points at either the 1- or 2-position, in response to the physical properties of the other endogenous or exogenous fatty acids available for complex lipid biosynthesis. The flexibility of this system is also manifested in its ability to incorporate significant amounts of exogenous fatty acids into the non-preferred position, when large quantities of that acid are present in the growth medium. Thus 9-18:1c, for example, is esterified quite readily into the 1-position when the 2-position, which is normally greatly preferred, becomes saturated with 9-18:1c. If this flexibility were lacking, this organism would not be capable of incorporating more than about 50 mole per cent of most exogenous fatty acids, as was discussed in a previous chapter of this dissertation.

There are two possible pathways for the synthesis of phosphatidic acid from alpha-glycerophosphate: in one pathway the first acylation occurs at the 1-position of sn-glycerol 3-phosphate to form 1-acyl-sn-glycerol 3-phosphate (1-acyl-GP), followed by the acylation at the 2-position (Pathway I); another pathway would utilize 2-acyl-sn-glycerol 3-phosphate as an intermediate (Pathway II). The relative importance of these two pathways *in vitro* or *in vivo* has yet to be studied in *A. laidlawii* B. In rat liver microsomes Pathway I has been suggested to be the major one (Tamai, 1972). In *E. coli*, however, there is still some controversy about which pathway is the more important (Ray *et al*., 1970; Okuyama and Wakil, 1973). The question as to whether only one enzyme, which acylates both the 1- and 2-positions of the glycerol moiety, or whether two different enzymes are involved, has not been addressed in *A. laidlawii* B. The existence of at least two different enzymes has been suggested in *E. coli* by the finding of two different temperature-sensitive mutants which appear to affect different acylation steps (Cronan, *et al*., 1970; Hechemy and Goldfine, 1971). It is also possible that more than one enzyme is responsible for each acylation of a single position, although it seems unlikely for *A. laidlawii* B because of its small genome size and simple fatty acid biosynthetic pattern.

The position-specific esterification of *alpha*-glycerophosphate to phosphatidic acid would seem to be the most economical and simplest mechanism by which an organism could insure proper asymmetric fatty acid positional distribution. This pathway has been studied *in vitro* in order to confirm that it is indeed responsible for the fatty acid positional specificity observed *in vivo*. However, the results of *in vitro* experiments usually do not fully mirror the situation *in vivo* (Hill *et al.*, 1968; Okuyama and Lands, 1972; Okuyama and Wakil, 1973). Okuyama and Lands (1972) have shown that the apparent *in vitro* specificity of monoacylglycerophosphate acyltransferase in rat liver microsomes is variable, depending upon the concentration of the acyl-acceptor, and have indicated the importance of experimental conditions on the *in vitro* specificity. Whether acyl-ACP or acyl-CoA is the true substrate *in vivo* is not known, and this might also create some discrepancy between *in vivo* and *in vitro* experiments.

Unlike *A. laidlawii* B, the higher organisms, as well as *E. coli*, deacylate and reacylate the phospholipids, and these fatty acid exchange reactions may be responsible for at least part of the apparent specific positional distribution of fatty acyl groups in phospholipid molecules (Lands and Hart, 1965; Van Deenen *et al.*, 1967; Yamashita *et al.*, 1973; Okuyama, 1969).

Almost nothing is presently known about the molecular basis for the fatty acid positional specificity described in this dissertation. Further progress in this area must await the development of a well-characterized, fully-functional cell-free system which accurately reflects the specificity of the enzyme system as it functions in this living organism. I hope that the experiments described in this and preceding chapters, in addition to being useful and significant in themselves, will provide information which will be useful in the development and utilization of appropriate in vitro model systems.

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